Directed growth and selective differentiation of neural progenitor cells using a synergistic combination of topographical and soluble cues

by

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ABSTRACT

The purpose of this study is to develop strategies to facilitate nerve regeneration using a synergistic combination of guidance cues. We are investigating the cellular mechanisms of development using adult rat hippocampal progenitor cells (AHPCs) and have demonstrated that manipulating a combination of physical, chemical and biological cues can lead to oriented growth and alignment of astrocytes and neural progenitor cells and can influence the progenitor cell differentiation. To provide physical guidance, micropatterned polystyrene (PS) substrates were fabricated and chemically modified with poly-\(L\)-lysine (PLL) and laminin. Rat postnatal type-1 astrocytes or AHPCs cultured on these substrates align along the grooves of the patterned surface. AHPCs appear highly elongated and often extend their processes in the direction of the grooves and along groove boundaries. To further explore the outgrowth and differentiation of the AHPCs, we have integrated the physical guidance cues along with the biological influence of astrocytes. AHPCs co-cultured in contact with astrocytes preferentially acquired neuronal morphology, with nearly double the percentage of cells expressing class III \(\beta\)-tubulin (TuJ1) on the micropatterned half of the substrate, as opposed to the planar half of the substrate, or compared to those growing in the absence of astrocytes. This indicates that substrate three-dimensional topography, in synergy with chemical (laminin) and biological (astrocytes) guidance cues, facilitates neuronal differentiation of the AHPCs. Through multi-dimensional cell-cell and cell-matrix interactions, this environment provided biological and spatial control over differentiation enhancing neuronal differentiation and promoting neurite alignment on topographically different regions of the same substrate. In this environment, the aligned astrocytes may present discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both. Using a non-contact co-culture system, it was determined that astrocyte-derived soluble factors can enhance neurite outgrowth and induce neuronal differentiation of the AHPCs with significantly more cells immunoreactive for TuJ1 in the non-
contact co-culture system than in the contact co-culture system. Therefore, soluble cues may have had a stronger influence on neuronal differentiation and neuritic extension compared to the contact mediated factors or the combination of soluble and contact mediated factors that were presented by the monolayer of aligned astrocytes. The results also point to the potential role of localized concentration of these factors within the microgrooves as a reason for the differences in differentiation on micropatterned and planar substrates in the contact studies as opposed to the non-contact co-cultures. This research provides insights into mechanisms of neural stem cell differentiation as well as a foundation for the development of a promising nerve regeneration strategy that incorporates a synergistic combination of cues for guided central nervous system repair following injury.
CHAPTER 1

INTRODUCTION AND SPECIFIC AIMS OF RESEARCH

1.1 Micropatterned Polymer Substrates for Regeneration in the Central Nervous System

Adult central nervous system (CNS) injury is typically followed by neuronal degeneration, cell death, and the breakdown of synaptic connections. It is believed that the adult mammalian CNS is not capable of self-repair or regeneration of the correct axonal and dendritic connections [1, 2]. It is established that fish, amphibians, mammalian peripheral nerves as well as developing central nerves respond differently to an injury than the adult mammalian CNS. In these systems, functional axons can regrow after they have been damaged [1]. However, currently, in the mammalian CNS, there is no treatment for the restoration of nerve function due to the intricate series of events that must take place in order for regeneration to occur.

Axonal regeneration in the CNS is limited by inhibitory influences of the glial and extracellular environment [3-5]. Consequently, regenerating axons in the CNS cannot reach synaptic targets and reestablish their original connections. Experimental strategies employing neural stem cell (NSC) transplantation hold great promise for repairing the injured and diseased CNS. NSCs are multipotent cells capable of self-renewal [6, 7]. NSCs, as well as neural progenitor cells (NPCs), which have more limited capacities in terms of growth and differentiation have been isolated and expanded from the developing and adult CNS in a variety of mammalian species, including humans [7-9]. These cells have been cultured successfully in vitro and differentiate into neurons and glial phenotypes [6-8, 10-13].

Efforts have been made in vitro to elucidate the specific external signals that control stem cell fate in vivo, known as the stem cell microenvironment, or ‘niche’ [6, 14, 15]. Studies have demonstrated that the fate of differentiating stem cells depends on direct cell-to-cell and cell-to-extracellular matrix (ECM) contacts and involves a complex “cocktail” of growth factors, signaling
molecules, and ECM proteins [16-18]. Furthermore, astrocytes have been studied as influential components of an ‘instructive’ stem cell niche [19]. Recent studies have shown that astrocytes induce neurogenesis in vitro of both adult neural [14, 20, 21] and embryonic precursor cells [22]. Although, astrocytes may be providing instructive cues to the precursor cells through a variety of signals, it has further been demonstrated that having cellular contact with an astrocyte is sufficient to promote neurogenesis of stem cells in culture [14, 19].

There are multiple factors in the microenvironment directly surrounding cells that can induce and maintain their functional stability. Elucidating which factors are involved and how they interact with cells is a principal aim of tissue engineering. To study the effects of the microenvironment on cell growth and differentiation, microfabrication technology has been applied to the design of substrates having specific architectures. This is known as ‘cellular micropatterning’ [23]. These culture environments are designed to encourage isolated cells to mimic their in vivo function. Cell growth can be controlled at the cellular level through the fabrication of microgrooves and other patterns on substrate surfaces [24]. Various polymeric biomaterials have been used to fabricate substrates incorporating physical guidance mechanisms such as these to provide instructive environments for neurite outgrowth [25-28].

The overall objective of this research project was to create a guidance substrate to facilitate directed growth and differentiation of NSCs with potential for aiding in CNS regeneration. Combining the biological influence of astrocytes along with physical and chemical guidance cues has the potential to generate a supportive environment for eliciting regeneration and restoring function in the injured or diseased CNS. Such an environment incorporating multiple guidance cues to direct the lineage of endogenous or engrafted CNS-derived precursor cells offers opportunities to elucidate the mechanisms behind efficient stem cell - associated repair of the CNS, specifically repair of the optic nerve. The ability of the CNS to transmit signals to the brain is severely hampered when retinal ganglion cells or the optic nerve are damaged by disease or injury. In an effort to provide an interface
between microelectrode components such as retinal prosthesis and the brain, this research was undertaken to provide an initial design and characterization of an engineered substrate onto which NSCs and microelectrodes could potentially be integrated.

The initial objective of this research was the fabrication of a micropatterned polymer substrate that would allow physical guidance of neural cells. From this substrate, a novel system was established that enabled the incorporation of chemical and biological guidance cues for the investigation of the growth and differentiation of neural cells such as astrocytes and NPCs. Specific aims for this research were as follows.

Specific Aim 1: To stimulate the oriented growth of astrocytes in vitro using physical and chemical guidance cues.

Physical and chemical mechanisms that direct and guide astrocyte adhesion and alignment in vitro were investigated. Micropatterned polystyrene substrates providing physical guidance were chemically modified with laminin, an ECM protein influential to cell migration, outgrowth, morphology and adhesion [29-31]. Laminin was adsorbed to the polystyrene substrate contributing substrate-associated factors for adhesion and directed outgrowth. The behavior and morphology of astrocytes on these substrates were extensively explored in order to optimize conditions for controlling their growth and subsequently use the astrocytes to influence differentiation and outgrowth of the AHPCs.

Specific Aim 2: To examine the influence of physical and chemical guidance cues on NPC growth and differentiation.

The effect of physical and chemical cues on the expansion, differentiation and outgrowth of the adult hippocampal progenitor cells (AHPCs) was explored. Micropatterned polystyrene substrates providing physical guidance were chemically modified with poly-L-lysine and laminin contributing
substrate-associated factors for adhesion, differentiation and directed outgrowth. The orientation and
differentiation of AHPCs on these substrates were evaluated. This established a control for Specific
Aim 3.

Specific Aim 3: To determine the influences of a synergistic combination of physical, chemical and
biological guidance cues on NPC growth and differentiation. This was done using:
(a) a contact co-culture system of astrocytes and AHPCs and
(b) a non-contact co-culture system of astrocytes and AHPCs.

To explore the outgrowth and differentiation of the AHPCs, the biological influence of
astrocytes was integrated with the physical and chemical cues. AHPCs were co-cultured on top of
near confluent monolayers of astrocytes on the patterned substrates. The dynamic microenvironment
created by these contact co-cultures was used to investigate mechanisms of AHPC differentiation,
including (1) the role astrocyte-derived factors played in differentiation and (2) how spatial control
provided by the micropatterned polymer substrate affected the manner in which these factors were
presented to the AHPCs.

To further investigate the astrocyte-derived factors in the multi-dimensional environment
described above, a non-contact co-culture system was designed utilizing a Transwell® semi-porous
membrane insert to separate the astrocytes from AHPCs cultured on micropatterned substrates in the
same well. These experiments were performed in an effort to identify the optimal combination of
signals creating both biological and spatial control over differentiation. This involved an examination
of whether the factors responsible for the selective differentiation of the AHPCs in the co-cultures
were contact-mediated or soluble or both.

The physical, chemical and biological cues provided by the polymer guidance substrates were
characterized and optimized to generate a dynamic environment for specific cellular behavior such as
the directed growth and selective differentiation of NPCs. The results of this research provide insights
into mechanisms of NSC differentiation as well as a foundation for the development of a promising regeneration strategy that incorporates a synergistic combination of cues for guided CNS repair following injury.

1.2  Dissertation Organization

This dissertation is organized in the following manner. Chapter 2 provides a comprehensive literature review. The remainder of the dissertation is organized such that each remaining chapter fulfills a specific aim of the research. Specific Aim 1 is discussed in Chapter 3 and is a manuscript published in *Biomaterials* (2004). Specific Aims 2 and 3a are discussed in Chapter 4 and is a manuscript that is published in *Biomaterials* (2006). Specific Aim 3b is discussed in Chapter 5, which is currently a manuscript in preparation. Chapter 6 and 7 detail the overall conclusions and the future directions for this research, respectively.
1.3 References


CHAPTER 2

LITERATURE REVIEW

2.1 The Central Nervous System and the Optic Nerve

2.1.1 The mammalian central nervous system

The mammalian central nervous system (CNS) is composed of the spinal cord and brain, including the medulla oblongata, the pons, the cerebellum, the midbrain, the diencephalon and the cerebral hemispheres. The CNS consists of tracts involving nerve fibers that are either myelinated or nonmyelinated. Bundles of nerve fibers, or axons, deliver sensory information to CNS and carry motor commands to the periphery.

There are two distinct classes of cells in the CNS. They are nerve cells and neuroglial cells. Nerve cells, or neurons, collect information from the environment directly or from other neurons and transmit this information to target cells, specifically other neurons in the CNS. Neuroglial cells surround nerve cell bodies and axons. They provide structural support, function in repair processes, provide isolation and insulation of groups of neurons from each other, are involved in metabolic functions and guide the migration of growing axons during development [1]. Neuroglial cells also may regulate neuronal shape and synaptic connectivity [2].

2.1.1.1 Neurons

Neurons of the CNS can appear morphologically different, yet they all have the same functional organization allowing collection and transmission of information. Most have several main features, including a cell body and two types of processes, or nerve fibers, called the dendrites and the axon (2.1). The diameter of the cell body is 50 μm or larger. It is the center of metabolic activity in the cell and contains the nucleus and the rough and smooth endoplasmic reticulum. Dendrites receive information and axons transmit information to other cells. Dendrites increase the surface area of
cellular communication as they branch out receiving signals from hundreds, sometimes thousands, of other neurons. The axon is capable of conveying electrical signals over distances from 0.1 mm to 2 meters. Compared to the cell body, most axons in the CNS are very thin ranging between 0.2 and 20 \( \mu \text{m} \) in diameter. Lipoprotein sheaths of myelin provided by a class of glial cells known as oligodendrocytes insulate many axons (myelinated axons). Synapses are specialized terminals where the axon terminals of one neuron (presynaptic neuron) transmit signals to the dendrites of another neuron (postsynaptic neuron). Neurotransmitters released from vesicles at the
presynaptic terminal send signals across a synapse to the postsynaptic terminal [1]. Specialized points of synaptic contact are made between neurons determining the function of particular neural networks organized within the CNS.

Neuronal structure provides valuable information about the function of the neuron. The anatomical location of the neuron, its size, origin, and the destination of the axons and dendrites are important features. Connections made with the neuron are also vital in determining neuronal function [2]. The number of processes that a neuron has distinguishes one neuron from another. Bipolar and multipolar are two general classifications for most neurons in the CNS. Bipolar cells, such as those found in the retina, have two processes, the dendrite and the axon, that have specialized functions. Multipolar cells have an axon and many dendrites. They are the most common neurons in the CNS and are found in the spinal cord, hippocampus, throughout the cerebral cortex and cerebellum.

Neurons are also classified by their function. In the mammalian nervous system, afferent neurons carry information toward the CNS from the periphery and motor (or efferent) neurons are motor fibers leading away from the brain and spinal cord [4]. A third class of neurons, known as interneurons, are responsible for relaying or projecting information over long distances as well as within local circuits [1].

2.1.1.2 Glial Cells

Two principal classes of glial cells existing in the CNS are astroglial cells (astrocytes) and oligodendrocytes (Figure 2.2). There is a great deal of information on these macroglial cells and their role in structural and functional support of axons in the central nervous system. However, this discussion will focus mainly on astroglial cells (specifically type 1 astroglial cells) as they are the primary concern in this research.

The oligodendrocytes are small cells with few processes that are responsible for insulating axons by wrapping their processes around them concentrically forming a myelin sheath. These cells
are similar to Schwann cells, the ensheathing and myelinating cells of the peripheral nervous system (PNS). However, a single oligodendrocyte enwraps several axons with its plasma membrane whereas in the PNS, one Schwann cell envelops one axon. The sheath is interrupted at regular intervals by gaps, called the nodes of Ranvier. Electrical excitation is confined to the nodes of Ranvier. Nerve impulses, or action potentials, are propagated quickly and efficiently as they travel along the axon jumping from node to node [5]. Oligodendrocytes also surround neuronal cell bodies providing support.

Astrocytes are the most numerous of the glial cells and have multiple functions in the CNS. These cells take on a variety of morphologies. Typically, astroglial cells have cell bodies that are polygonal or circular in shape usually with many long processes. In culture, astroglial cells are flat, adherent cells. They have a cobblestone-like appearance and lack polarity at confluence. A minority

Figure 2.2: Neuroglial cells in the central nervous system. A) Protoplasmic astrocytes. B) Fibrous astrocytes. C) Oligodendrocytes [3].
of these cells can also appear irregular in morphology and exhibit one or more long processes. *In vivo*, their appearance closely resembles neurons having polarity and one or more thick processes. Astrocytes are in close contact with neurons and endothelial cells from capillaries and can also envelop synaptic terminals [6] (Figure 2.3). These glial cells are connected to each other through tight junctions, or gap junctions. These specializations allow the passage of small molecules between the cytoplasm of neighboring cells. Due to their position within the CNS, astrocytes have the capability of establishing communication through signaling pathways between neurons, between astrocytes, and between neurons and capillaries [7].

![Figure 2.3: Schematic of one synapse enveloped by filopodial extension of one astrocyte [8].](image)

There are various types of astrocytes present in the mammalian brain. Based on the length of the processes of these cells, astrocytes have been placed into two categories: fibrous astrocytes, which have extensive intermediate filaments and protoplasmic astrocytes containing fewer filaments (Figure 2.2 above). A third subclass of astrocytes, reactive astrocytes, appears following injury to the brain. These classifications are typically used to describe astrocytes *in vivo*. Brain cell cultures have revealed two glial cell lineages. These two types of astrocytes are called type-1 and type-2 astroglia [9]. In cell suspensions of the rat optic nerve, it was discovered that type 1 astrocytes appear prenatally and type 2 astrocytes appear at the beginning of the second postnatal week [10].
Morphology, antigenic phenotype, and response to growth factors are used to distinguish these cells from each other [9]. The intermediate filaments of astroglia are composed of glial fibrillary acidic protein (GFAP). The presence of this protein is used as a marker for observing and quantifying astroglial cells. A2B5, a monoclonal antibody directed against gangliosides, is used to differentiate between type-1 and type-2 astrocytes. This antibody labels type-2 astrocytes but not type-1. In addition to the optic nerve, there have been cells of type-1 astroglia and type-2 astroglia observed in cultures of cerebellum [11] and cerebral cortex [12].

Astrocytes are known primarily to function in supporting roles providing structural support and nutrition to neurons and other cells in the CNS. They form continuous sheets called limiting membranes as they extend processes to the nerve surface and are important in the formation of the blood-brain barrier between the CNS and other tissues. Astrocytes also express a high density of potassium ion (K⁺) channels. Due to this characteristic, they are able to buffer extracellular potassium that accumulates when neurons fire action potentials repeatedly and take in excess neurotransmitters released by neurons [13]. *In vivo* and *in vitro* studies have been undertaken revealing that astroglial cells express receptors and function in neurotransmitter uptake. They have also been proposed as a source of trophic factors necessary for neuronal survival [14]. It has been shown that astroglial cells function in neuronal guidance [15]. More recently, Song and colleagues reinforced an emerging view that astrocytes have an active regulatory role in the CNS. Their findings presented strong evidence that astroglia promote neurogenesis from adult neural stem cells [16]. Invaluable experimentation performed in the last two decades has revealed that astrocytes are much more complex than previously thought and are capable of significant influence on neuronal activity.

2.1.1.3 Astroglial-Neuronal interactions

Neurons and astroglial cells interact extensively within the mammalian CNS. Among the functions attributed to astrocytes are the support of the proliferation, survival, and maturation of
developing neurons. Astrocytes are thought to guide migrating neuronal precursors and advancing growth cones to their destination [17]. They provide structural, metabolic, trophic as well as tropic support for neurons [1]. Astrocytes regulate neuronal activity through the release of soluble trophic factors, such as glutamate [18, 19]. Haydon reported recently that astrocytes are capable of integrating neuronal inputs and modulating synaptic activity [13, 20]. In the past, it was believed that the role of astrocytes was to provide a passive, supportive mechanism for neurons. They are now believed to take on an active, regulatory role [16, 21].

Type-1 astroglial cells express a variety of functional neurotransmitter receptor and uptake systems. Type 1 astroglia have been shown to activate second messenger systems including cyclic AMP [22], cyclic GMP [23], and intracellular calcium [24]. These receptor systems also regulate ion channel opening [25]. Voltage dependent ion channels have been demonstrated in astroglia as well [26]. There has been much evidence showing that astrocyte receptors participate in neurotransmitter uptake. Mammalian astrocytes from neonatal rat brain were identified as taking up serotonin by a sodium-dependent mechanism with high-affinity [27]. Astrocytes have been able to respond to amino acids, amines, peptides, purines, and prostaglandins [28]. It has been demonstrated that astrocytes respond to a variety of synaptically released neurotransmitters including glutamate [29], noradrenaline [30], histamine [31], acetylcholine [31], ATP [32, 33], and γ-aminobutyric acid (GABA) [34]. These transmitters induce elevations of astrocytic Ca\(^{2+}\). Astroglial receptor systems are not only involved in response to transmitters, but the integration of inputs and signaling leading to transmitter release.

Astroglia function in the induction and stabilization of CNS synapses. There is in vitro evidence that astrocytes might play an important role in determining how well-connected neurons end up by direct influence on their synapses. Pfrieger and Barres reported that culture media from astrocytes caused a tenfold increase in the synaptic activity of RGCs [35]. Work by Ullian and colleagues demonstrated that RGCs that are cultured in the presence of astroglia increased in synaptic
number significantly and the synapses were also more mature. They revealed that in vivo generation of synapses occurs alongside the development of glia. These results are in agreement with what was previously observed about synapse number in vivo in the superior colliculus [36]. Nagler et al. [37] has recently reported that synapse formation in purified CNS neuron cultures is induced by a soluble glia-derived factor. Mauch et al. [38] demonstrated just months later that this factor was cholesterol in a complex with lipoproteins containing apolipoprotein-E. Blondel et al. [39] has also recently presented that a soluble astrocyte-derived factor promotes synapse formation and enhances glutamate sensitivity in cultured hippocampal neurons. Furthermore, there is significant evidence that synapses and the astroglia associated with them form tripartite synaptic structures in the CNS. Synaptic transmission is modulated by such structures in a “feedback” manner [40].

Type-1 astroglial cells exhibit growth factor secretion. Astrocytes release a variety of soluble factors many of which are involved in neuronal signaling, including glutamate [19, 20, 41], ATP [32, 42], and β-chemokines [43]. Cultured glial cells from various regions of the brain synthesize and secrete nerve growth factor (NGF) [44]. In the optic nerve, type-1 astroglia secrete growth factors that induce the proliferation of O-2A progenitor cells [45]. In vitro experimental evidence has suggested that platelet-derived growth factor (PDGF) is responsible for this stimulation of O-2A proliferation [46, 47]. Growth factors released from astroglia are also involved in the differentiation of O-2A progenitors to type-2 astroglia. It has been deduced that ciliary neurotrophic factor (CTNF) or a closely related protein plays a role in inducing type-2 astroglia differentiation [48]. Furthermore, various glycoproteins and proteoglycans secreted by astrocytes into the extracellular matrix (ECM) have been shown to influence axonal outgrowth. Astroglia produce and express receptors that bind a number of neuron-adhesive ligands, including laminin [49], fibronectin [50], and members of the cell adhesion molecule (CAM) class of proteins, including NCAM [51] and related proteins. Laminin, a substrate adhesive molecule, is produced by astroglia and secreted into ECM. This molecule has been shown to promote extensive neurite outgrowth [52]. Chondroitin sulfate proteoglycan (CSPG), an
ECM molecule associated with astroglia, was reported by Snow et al. to direct the growth of retinal ganglion cell processes in the rat retina [53]. It has been demonstrated in vitro that astroglia release factors in the culture medium that are capable of enhancing growth and prolonging the survival of hippocampal neurons [54] and inducing morphological differentiation in neuroblastoma cells [55]. Astroglia provide supporting elements not only through the release of soluble factors into culture medium but through cell-to-cell contact as well [14].

Astroglia take an active part in neuronal guidance. It has been presented that astroglia play a major role in neuronal migration [15]. Astroglia influence the direction of axonal outgrowth by providing a pathway for axonal outgrowth or by becoming barriers to prevent inappropriate axon pathway growth in certain locations. Astrocytes function actively in neurite extension and patterning [56, 57]. In vitro and in vivo, evidence has been reported suggesting that neurite pathfinding can be controlled solely by astrocytes in the developing CNS [57, 58]. CSPG has been described as forming barriers to direct growth of retinal ganglion cell axons in the developing retina [53]. Retinal ganglion cell differentiation has also been demonstrated using similar ECM molecules produced by astroglia [59]. Astrocytes take on these active roles through the release of cytokines, proteases, and protease inhibitors [60, 61]. They also have a variety adhesion molecules on their surface that are influential in axon growth and produce various extracellular matrix molecules [57, 62, 63]. The proper combination of astroglia-associated permissive and non-permissive guidance cues produced by astrocytes in the precise locations direct neurites to their destinations. It is thought that astrocytes regulate the expression of these particular guidance cues within the CNS [64]. Changes in chemical composition of the extracellular fluid produced by glial or neuronal stimulation mediate interactions between optic nerve axons and astroglial cells. Certain interactions have been characterized as lasting for brief periods involving changes in K⁺ or H⁺ concentrations, or alterations involving small molecules, including glutamate or ATP, while others involve much longer periods and involve larger signaling molecules such as peptides or proteins [65].
Furthermore, there has been *in vitro* and *in vivo* evidence reported demonstrating that, in response to CNS injury, astroglial cells exhibit considerable plasticity and that neuron-glia relationships regulate what occurs in the CNS after injury [66]. Astrocytes produce both soluble and membrane-associated factors that influence CNS function and provide a surface that may change the “sprouting” of axons following injury [21, 67]. Recently, Song and colleagues revealed that hippocampal astrocytes instruct the neuronal fate commitment of adult neural stem cells [16].

2.1.2 *The optic nerve*

The optic nerve (ON) consists of the long nerve processes or axons of retinal ganglion neurons projecting from the eye to the brain. These axons reside in narrow extracellular spaces alongside the cell bodies and processes of glial cells [65] (Figure 2.4).

![](image)

**Figure 2.4:** A model of how the three types of glial cells are arranged in the adult rat optic nerve [5].

Undifferentiated cells, including glial progenitors, are also retained by the optic nerve [68-70]. A wide range of interactions exists between the retinal ganglion cells (RGC) axons and glial cells found within and along the ON. Despite the anatomical simplicity of the optic nerve due to the lack of neuronal cell bodies, a variety of interactions occur between axons and astroglial cells in this pathway both *in vivo* and *in vitro* (Figure 2.5). Neuron-astroglial interactions provide a permissive environment for the prevention of RGC death and restoration of ON function following injury.
2.1.2.1 Organization of the visual system

Vision is an integral part of neural function. The eye consists of a pupil that adjusts lens aperture, a lens that focuses light on the retina, and the retina, or the neural layer of the eye, where photoreceptors are present. The lens is made of many layers of cells that provide translucency. These layers also have viscoelastic properties that are important in focusing, or adjusting lens thickness. The retina receives visual images. It converts the image into nerve impulses and facilitates feature analysis of the image. Feature analysis and visual interpretation proceed progressively as signals from the retina are passed to other visual centers in the brain.

The retina is the innermost layer of the eye. It is responsible for receiving light and consists of many retinal cells. There are several layers of cells that make up the retina. The cells in these layers include photoreceptor cells (rods and cones), bipolar cells, ganglion cells, and pigment epithelial cells (Figure 2.6). The pigment epithelium is the most outer layer of the retina and is a single cell-layer of melanin-containing cells. The neural cells are found just outside of this layer. Each cell type forms a cellular layer and they are linked by synapses. The outer segments of the rods and cones form the photoreceptive layer. The cell bodies of the rods and cones make up the outer nuclear layer. The axon
terminals of the rods and cones synapse with the dendrites of the bipolar cells (sensory neurons) making up the outer plexiform layer. The nuclei and perikary of the bipolar cells form the inner nuclear layer. Axon terminals of the bipolar cells synapse with the dendrites of the ganglion cells forming the inner plexiform layer. The innermost layer of the retina consists of the cell bodies of the ganglion cell, the ganglion cell layer. The axons of the ganglion cells form the optic nerve fiber layer. Light focused by the lens first passes through the nerve cells of the retina to reach the rods and cones at the periphery of the retina. Visual information then moves through the retinal layers, from the photoreceptive layer toward the ganglion cell layer. Finally, the optic nerve transmits visual

Figure 2.6: The anatomy of the human eye and a cross-section of the human retina [71].
information from the retinal ganglion cells through the optic disk to three nuclei of the visual system. The axons of RGCs terminate in (1) the lateral geniculate nucleus (thalamic nucleus) of the brain for projection of fibers to the visual cortex, (2) the rostral colliculus for the visual and pupillary light reflexes (pupillary dilation), and (3) the pretectal nucleus for the pupillary light reflex (pupillary constriction).

The optic nerve becomes the central tract along which visual messages are carried out of the eye along the retinal ganglion cell axons. The optic nerves from each eye join its opposite at the optic chiasm. At the chiasm, optic nerve fibers from the nasal halves of each retina cross. The fibers in the temporal halves remain uncrossed. The optic tracts, which extend from the chiasm to the thalamus, contain fibers that convey visual information from both eyes. If there is injury to one optic nerve, the result is total blindness in that particular eye. If the optic tract is damaged on one side, the result is partial blindness in both eyes.

2.1.2.2 Optic nerve injury and regeneration

It is believed that the adult mammalian central nervous system (CNS) is not capable of self-repair or regeneration of the correct axonal and dendritic connections [72, 73]. Adult CNS injury is typically followed by neuronal degeneration, cell death, and the breakdown of synaptic connections. It is established that fish, amphibians, mammalian peripheral nerves as well as developing central nerves respond differently to an injury than the adult mammalian CNS. In these systems, functional axons can regrow after they have been damaged [72]. The adult mammalian optic nerve is thought to be incapable of significant self-repair or regeneration under normal physiological conditions. Presumably injury to the optic nerve, including transection, elevated intraocular pressure, ischemia, and compression is permanent and devastatingly irreversible. Without any capacity for repair, it is believed that those with damage to the ON will not be able to restore their sight. This belief stems from three major assumptions: (1) the mammalian RGC will die once its cell body or axon has been
injured; (2) an injured mammalian RGC whose axon has degenerated cannot be induced to extend a new axon; and (3) even if induced to regenerate, the regenerating axon cannot be guided back to its original target in the CNS. Successful regeneration depends on reinduction of necessary intracellular mechanisms as well as the synthesis of proteins for support of axonal growth. Furthermore, the reactivation of long-range pathfinding functions present during development must occur, and proper conditions must be met for re-establishment of appropriate connections in the target areas of the brain [74]. Studies have provided evidence that, under certain conditions, a lesioned neuron can survive axotomy, re-extend a regenerated axon and reform patterned connections within the CNS [75].

Following axotomy of the optic nerve, it has been shown that there is a transient state of regeneration followed by a period of retrograde degeneration of ganglion cells. Optic nerve transection results in a cascade of inflammatory events that lead to ganglion cell terminal degeneration followed by phagocytosis of somata in the retina [76-78]. After the axons of the optic nerve are severed, nearly all RGCs survive for about 5 days and then begin to die off. Approximately 90% of the RGCs undergo apoptosis, or programmed cell death, within two weeks [79, 80]. Microglia in the retina that are signaled by the axotomised ganglion cells help this self-destruction [81]. Once the normal programmed degeneration of RGCs following injury is interrupted, the surviving neurons must then re-extend axons within the CNS environment. Experiments have confirmed observations made by Cajal that the myelinated region of the ON (later verified as the myelin of oligodendrocytes) inhibits axon growth in the CNS [76, 82-84]. Therefore, in order for a surviving neuron to re-extend an axon along the CNS, the inhibitory molecules of the myelin must be neutralized.

Molecular methods have been shown to increase the survival of lesioned ganglion cells. Among these methods are injections of neurotrophic factors into the eye, including nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), and growth factors isolated from the peripheral nervous system, the in vitro application of peptides, the
intraocular injection of molecules inhibitory to the microglial activity, and the expression of the bcl-2 protein [85, 86]. Manipulating the external as well as the internal environment of the optic nerve has allowed the ganglion cell to survive axotomy. There seems to be a critical dependency on the availability of exogenous and endogenous neuronal trophic support. For nearly twenty years, optic nerve regeneration has been attempted in the laboratory at certain critical stages of CNS development and after injury, with the involvement of existing pathways using antibodies to neutralize inhibitory factors, the manipulation of peripheral nerve grafts, and the use of neural stem cells.

Surgical implantation of hybridoma cells continually secreting monoclonal antibodies (IN-1) has blocked inhibitory molecules in the area surrounding lesioned spinal cord [87]. These antibodies used in combination with other growth factors, such as BDNF [88], as well as the inactivation of myelin-associated glycoprotein (MAG) [89] have allowed lesioned RGC axons to regenerate significantly within the optic nerve. Gliosis also exhibits an inhibitory effect to axon growth in the CNS. Gliosis is the scar reaction that results in a massive proliferation of gliotic tissue surrounding the area where the CNS has been injured. This glial reaction is mediated primarily by astrocytes as they are attempting to keep pathogens out of the CNS in an effort to re-establish the blood-brain barrier [90]. Inhibitory molecules are also synthesized during glial scar formation, including chondroitin sulfate proteoglycan (CSPG) [53]. However, it has been revealed that the formation of the glial barrier presents trophic factors and has a role on reconstructing the blood-brain barrier as it isolates intact CNS tissue from secondary lesions [67]. As gliosis presents itself only at the site of injury, regrowing axons are able to grow in parts of the CNS tissue that have not been disrupted [91]. In the retina, there is minimal gliotic reaction with very little CSPG expression following injury [92]. Regenerating adult rat retinal segments have demonstrated axon extension after explantation onto a substrate consisting of Schwann cells [93] or immature astrocytes [94]. These results suggest that the retina may be a more favorable site for regeneration than other regions of the CNS.
It has been well established that peripheral nerve segments and Schwann cells exert trophic influences on survival and regeneration of axotomized ganglion cells [95-98]. Work by Videl-Sanz and colleagues [96] demonstrated regeneration of ON axons into and through a sciatic nerve graft and to the superior colliculus. Regeneration of ON axons through transection was also induced by Lazarov-Spiegler and colleagues by bathing a transected and subsequently reconnected mouse ON in a solution containing macrophages incubated with sciatic nerve [99]. Semipermeable acrylic copolymer tubes have been reported to bridge transections of the rabbit optic nerve using entubulization techniques [100]. The use of an electric field for regeneration of the optic nerve has also been investigated [101]. It has been revealed through much experimentation that the environment surrounding the retinal ganglion cell and its axon has been proven to be extremely important for the successful regeneration of the injured ON.

Directing a new axon to its correct target in the CNS has proven to be a major challenge in experimental strategies for ON regeneration. The process of synaptic connection and refinement must occur to ensure that the correct retinal ganglion cells are connected to the appropriate targets restoring retinotopic maps and, ultimately, sight [75]. In the opossum, it has been shown that at early stages of development that ganglion cells can regenerate across an incision made in the retina. Regenerating axons were also shown to respond to guidance cues within the optic chiasm [102]. The mechanisms behind the expression of the guidance cues in the adult visual pathway during regeneration are currently under much investigation. This research relies on the close association that exists between development and regeneration. Furthermore, neural stem cells have extraordinary potential to restore sight after injury to the ON. These cells are capable of replacing dead or damaged RGCS, providing the “enzymatic machinery” to correct metabolic defects, acting as sources of growth factor delivery to cells, or cellularly bridging disconnected cells [75].
2.1.3 **Neural stem cells in the adult central nervous system**

Neural stem cells are described as generating neural tissue or being derived from the neural system, having capacity for self-renewal, and they are multipotent or possess the ability to adopt a variety of cellular fates. Neural progenitors with more limited capacities in terms of growth and differentiation have been known to proliferate throughout life in a variety of mammalian species, including humans [103, 104]. Neural stem cells that have the potential to produce new neurons and glia are present in the adult mammalian CNS. These cells can remain in certain regions of the adult CNS after development even though neurogenesis no longer occurs in most areas after birth. Progenitor cells isolated from the subventricular zone as well as the dentate gyrus of the hippocampus have demonstrated proliferative neurogenesis into adult life. Cultures that have been initiated from these tissues have revealed both neuronal and glial-restricted progenitor cells as well as multipotent precursors [105, 106]. Palmer and colleagues revealed that the neural progenitors were able to differentiate into neurons after activation by fibroblast growth factor-2 (FGF-2). Furthermore, in the adult optic nerve, exposure to FGF-2 activated a latent neurogenic potential that is retained by precursors in this pathway. The results from this study supported the belief that neurogenesis may be restricted by the local environment cues in the developing and adult brain [16, 107]. This work confirmed that there are stem-like cells existing naturally that are capable of producing neurons within diverse tissues of the adult brain, such as the optic nerve, a structure that is separate from the neurogenic zones within the brain [108].

Neural stem cells are capable of long-term survival, migrate in response to injury cues, and are capable of differentiating along multiple CNS neural cell lineages. Stem cells integrate with host cells, will not initiate immune response or grow abnormally leading to tumor formation [75]. Neural stem cells have been isolated from adult, developing and embryonic brain [105]. Stem cells have also been found in the retina and ciliary body of embryonic and adult mice and rats, and human embryos [109]. They can be induced to differentiate into RGCs using the appropriate conditions [109-111].
Neural stem cell transplantation has been proposed as a method of repairing the diseased or damaged CNS through neuronal replacement. Gage and colleagues revealed that transplanted cells capable of proliferation and neurogenesis that were isolated and cultured from the adult rat hippocampus retain the capacity to generate mature neurons when grafted into the adult brain [112]. These cells have also been found to be capable of functionally integrating into the host hippocampal circuitry [113]. Neural stem cell transplantation has been attempted with neonatal and adult eyes [114, 115]. Transplanted characterized stem cells, such as adult hippocampal-derived neural stem cells, can be grafted back into the retina. Young and colleagues were the first to experiment with transplanting neural stem cells- derived from the adult rat hippocampus- into a diseased retina. In these studies, the researchers found that the neural stem cells morphologically integrated into the eye. They not only migrated to the right place and appear to take on the right characteristics, but they also show signs of trying to connect from the retina to the brain [114]. Kurimoto and colleagues had similar success with the injection of transplanted neural stem cells into retinas injured by transient ischemia [116]. Prior to the work of Van Hoffelen et al., the capacity of the retina to support transplanted neural stem cells had only been observed in diseased or damaged mature neural retinas [114, 116]. Van Hoffelen et al. has recently demonstrated that transplanting brain progenitor cells from the opossum into the developing and mature opossum eye led to survival and differentiation in vivo with extensive morphologic integration in the host retina [117]. It was revealed that these cells displayed characteristic morphologies of retinal neurons and that age of the host appeared to play a role in determining cell fate [117]. Neuronal replacement strategies, such as neural stem cell transplantation, that incorporate the insight gained from exploring cues that influence proliferation and differentiation of neuronal progenitor cells are valuable to the study of CNS, and more specifically, optic nerve regeneration and repair.
2.1.4 Adult neural stem cell-astroglial interactions

Local environments have a profound influence on the fate of adult neural stem cells. In the postnatal period in which the proliferation of progenitor cells progressively decreases, there is correspondence to a period of maturation of astrocytes. In the subventricular zone and the dentate gyrus of the hippocampus (the germinative zones of the brain), where proliferation of neuronal progenitors continues throughout adulthood, astrocytes are present that exhibit the morphology and phenotype of immature astrocytes. Alvarez-Buylla and Lim showed that in vitro direct cell-to-cell contact between the progenitors of the adult subventricular zone and astrocytes stimulated proliferation of progenitors [118]. In recent in vivo work by Alonso, it was revealed that astrocytes present in the germinative zones of the CNS provide a microenvironment that supports the proliferation of neuronal progenitors [119].

It has been further demonstrated that astroglial cells provide a permissive environment for neurogenesis. Song and colleagues recently established that factors from astrocytes increase the rates of neuronal fate commitment nearly six-fold and proliferation of progenitor cells twofold [16]. They have shown that neonatal as well as mature hippocampal astrocytes in the adult brain promote neurogenesis. Astroglial cells were observed as taking an active, regulatory role in neuronal production in the adult CNS. It was deduced that regionally specified astroglial cells have the potential to function as cues for neural stem cell differentiation of FGF-2 dependent, adult-derived stem cells [16].

2.2 Guidance Strategies for Central Nerve Regeneration

CNS axons typically do not regenerate due to a lack of support by the endogenous environment following injury. In the CNS of mammals, axonal regeneration is limited by inhibitory influences of the glial and extracellular environment [120]. Myelin-associated inhibitors of neurite
growth, astrocytes, oligodendrocytes, oligodendrocyte precursors and microglia migrate to the injury site making the environment non-permissive for axonal regrowth. In the distal axonal segment, degeneration is slower in the CNS compared to the PNS, and inhibitory myelin and axonal debris are not cleared away as quickly. The axons that survive axotomy are surrounded by unfavorable glial reactions at the lesion site, known as the glial scar. Neurons cannot regenerate beyond the glial scar where axonal outgrowth is essentially stopped [121]. Proximal axons initially demonstrate a spontaneous attempt to regenerate, but the surrounding environment rapidly hinders growth [122]. Consequently, regenerating axons in the CNS cannot reach synaptic targets and reestablish their original connections. Mechanisms for removing or neutralizing the inhibitory components of cellular debris cannot be found in the CNS. However, severed mammalian CNS axons will regrow in more permissive environments [97, 123, 124]. They are able to recognize target areas and to re-establish functional synapses with target neurons [125, 126]. There has been much recent evidence that suggests that the mature CNS is a less hostile environment for regeneration than was previously thought. If the axons can transverse the injury site, there is possibility of regrowth in the unscarred areas and of functional recovery [127, 128]. The use of cell implantation and replacement therapies involving neural stem [112-114, 117], Schwann [129-131] and olfactory ensheathing cells [132], scaffolds [127, 133-138], as well as delivery of growth factors [139, 140] have provided greater potential for production of new neurons and the repair of injured CNS regions.

2.2.1 Enhancing Nerve Regeneration using Entubulation

For successful regeneration, damaged axons must be prevented from dying, the sprouting axons from the proximal nerve stump must extend axons toward their targets, across the injury site, into the distal nerve stump and make synaptic connections to the correct target regions. Common repair techniques facilitating regeneration include grafting using natural materials and entubulation using nerve conduits or scaffolds. These methods connect proximal and distal nerve stumps using a
synthetic or biologically-derived conduit. Such conduits optimize regeneration by allowing for both physical and chemical guidance and reducing cellular invasion and scarring of the nerve. Entubulization minimizes unregulated axonal growth at the site of injury by providing a distinct environment, and allows for diffusion of trophic factors emitted from the distal stump to reach the proximal segment, which enhances physiological conditions for nerve regeneration. The transplantation of tissue engineered nerve conduits based on polymers and alternative methods to engineer an artificial environment to mimic natural physical and chemical stimulus promotes nerve regeneration and minimizes difficulties associated with grafting.

Tissue engineered nerve conduits based on polymers have been created for implantation mimicking the three-dimensional and biological environment that is necessary for enhanced regeneration. While bridging the gap between nerve segments, these conduits can preserve neurotropic and neurotrophic communication between the nerve stumps, repel external inhibitory molecules and provide physical guidance for the regenerating axons similar to the grafts [141] (Figure 2.7). The spatial cues provided by conduits also induce a change in tissue architecture cabling cells within the microconduit [142]. Polymers are being extensively investigated to help facilitate nerve regeneration and provide physical and chemical stimulus to regenerating axons [143, 144]. These materials vary in composition from entirely synthetic to naturally-derived biomaterials. Synthetic conduits are fabricated from metals and ceramics, biodegradable (i.e. poly(esters), such as poly(lactic acid) [145-147], poly(lactic-co-glycolic acid)[147, 148], and poly(caprolactone)[149, 150], or polyhydroxybutarate [151]) and non-biodegradable (i.e. methacrylate-based hydrogels [152], polystyrene [153], silicone [154, 155], expanded poly(tetrafluoroethylene) or ePTFE (Gore-Tex®: W.L. Gore & Associates, Flagstaff, AZ)[156, 157] or poly(tetrafluoroethylene) (PTFE)[158]) synthetic polymers. These materials are especially advantageous because specific chemical and physical
Figure 2.7: Silicone-chamber model showing the progression of events during peripheral-nerve regeneration. After bridging the proximal and distal nerve stumps, the silicone tube becomes filled with serum and other extracellular fluids. A fibrin bridge containing a variety of cell types connects the two stumps. Schwann cells and axons processes migrate from the proximal end to the distal stump along the bridge. The axons continue to regenerate through the distal stump to their final contacts [141].

Properties can be readily changed depending on the application for which they are used. Such properties include microgeometry, degradation rate, porosity, and mechanical strength.

Biologically-derived materials include proteins and polysaccharides (i.e. ECM-based proteins including fibronectin [159], laminin [160] and collagen [161, 162], fibrin and fibrinogen [163-165], hyaluronic acid derivatives [166], and agarose [167]). Collagen has been the most widely used natural polymer [162]. These natural materials are biocompatible and enhance migration of support cells [168]. However, batch-to-batch variability needs to be considered when using biological materials such as these. Selecting the appropriate material for a particular application is an essential part of the scaffold design. There are also certain physical properties and that are most desirable for nerve conduits [169] (Figure 2.8). General requirements for scaffold design are typically followed,
which include being biocompatible, having a high surface area/volume ratio with sufficient mechanical integrity and having the ability to provide a suitable environment for axonal growth that can integrate with the surrounding neural environment. These biomaterial-based (synthetic or natural) conduits can also be environmentally enhanced with chemical stimulants, such as laminin and nerve growth factor (NGF), biological or cellular cues such as from neural stem cells as well as Schwann cells and astrocytes, the satellite cells of the peripheral and central nervous systems, and lastly, physical guidance cues.

2.2.2 Cell guidance: In vitro experimentation

Providing an instructive environment for damaged neural tissues that have suffered trauma is essential for the regeneration of the injured nervous system. In order to generate an environment that supports regeneration, physical, chemical, and biological manipulations must be made. Guidance cues must be presented that aid in control of nerve outgrowth and navigate neuronal growth cones to distant targets in vivo. “Intelligent” nerve conduits having the appropriate combination of such cues will provide insight into the mechanisms behind axon growth and regeneration in nervous system. These scaffolds allow permissive guidance cues to be precisely oriented enhancing regeneration and aiding in the repair of severed or injured neural tissue.

2.2.2.1. Physical Modifications: Microtexturing and Micropatterning

Scaffolds are extremely useful for evaluating nerve regeneration processes for many reasons. Among these is that the properties of these conduits can be physically altered to optimize nerve regeneration. The microtexture of the surface of the lumen within the conduit affects the outgrowth of neurons and regulates regeneration. Smooth inner surfaces allow the formation of an organized, discrete nerve cable having many myelinated axons. In contrast, inside rough inner surfaces, nerve
fascicles are dispersed throughout the lumen and unorganized resulting in little regeneration. When comparing

Figure 2.8: Properties of the ideal nerve guidance channel. The desired physical properties of a nerve conduit include (clockwise from top left): a biodegradable and porous channel wall; the ability to deliver bioactive factors, such as growth factors; the incorporation of support cells; an internal oriented matrix to support cell migration; intraluminal channels to mimic the structure of nerve fascicles; and electrical activity [169].

expanded microfibrillar poly(tetrafluoroethylene) (Gore-Tex®: W.L. Gore & Associates, Flagstaff, AZ) tubes having different internodal distances (1.5-10 μm) to smooth-walled, impermeable PTFE tubes, it was discovered that rougher the texture of the surface, the greater the spread of nerve fascicles [170]. Furthermore, the molecular and cellular makeup of the regenerating tissue is affected by the stability of the wall structure [146] and channel geometry [171].

In addition to texture, scaffolds can exert control over other aspects of the neural environment. The structure of these scaffolds can be precisely defined for a particular application. Relying on knowledge of structure and function of cells and tissues in the nervous system, specific biomaterial "architecture" can be created and applied to the reconstruction of tissue function. The
susceptibility of a cell to topographical structure is determined by the organization of the cytoskeleton, cell adhesion, and cell-to-cell interactions [172]. Cell growth can be controlled at the cellular level through the fabrication of microgrooves and other patterns on substrate surfaces [173]. The development of microfabrication and nanofabrication techniques involving photolithography and reactive ion etching has allowed precise control over patterned features using a variety of materials. Recent developments in manufacturing techniques have included the move from silicon-based fabrication to polymer-based biomaterial scaffolds and the creation of three-dimensional constructs based on success with two-dimensional fabrication methods. Isolating large numbers of individual cells and having control over their shape and distribution is extremely valuable in the analyzing functional changes in individual cells and their relationship with their environment in culture. Fabrication techniques producing substrates with various feature shapes and dimensions are used to study cell behavior and morphology in vitro before integrating similar techniques into a scaffold design. In the recent past, micropatterned biodegradable and non-biodegradable substrates have been developed using microfabrication and transfer patterning techniques [153, 174-176]. Microcontact printing techniques involving elastomeric polydimethylsiloxane (PDMS) stamps have been used to create adhesive islands for the control of cell shape, growth and function [177, 178]. Microfluidic patterning has also been used for developing topographical cues on substrates [179]. The effects of the microenvironment on cell behavior has been studied on different substrate materials, including Perspex [172, 173, 180], silicon wafers [181-183], quartz [184, 185], and non-biodegradable polymers such as polystyrene [153, 186] and biodegradable polymers, including poly (lactide-co-glycolide) and poly (DL-lactide) [175, 176].

Cell adhesion and proliferation has also been examined using various shapes and feature sizes with several neural cell types. Rectangular shapes [174, 187], hexagonal [182] as well as circular features [188] have been successful in controlling cellular behavior. In experiments using lithographically patterned quartz, hippocampal neurites grew parallel to deep, wide grooves but
perpendicular to those that were shallow and narrow. Neurites also grew faster in the favored direction of orientation and turned through large angles to align on grooves [187]. The shape and expression of a differentiated phenotype of retinal pigment epithelium (RPE) was controlled using octadecyltrichlorosilane (OTS)-modified glass micropatterned substrates [188]. Webb et al. [185] demonstrated that rat optic nerve astrocytes aligned on surface features as small as 100 nm depths with 260 nm pattern spacing on quartz discs. The oligodendrocyte lineage displayed a high degree of sensitivity to topography as well [185]. Schwann cell and neurite alignment has been demonstrated on micropatterned biodegradable substrates with evidence that groove depth affects the proportion of neurites aligned. Deeper grooves have a stronger effect on cellular behavior [174, 176]. Furthermore, these micropatterned biodegradable polymer films were inserted inside poly(D, L-lactide) (PDLA) conduits. The micropatterned surfaces were pre-seeded with Schwann cells in order to provide guidance to axons at the cellular level. Over 95% alignment of the axons and Schwann cells was observed on the micropatterned surfaces with laminin selectively attached to the microgrooves [174].

Mechanisms of contact guidance as well as the intracellular distribution of cytoskeletal elements such as microtubules, microfilaments, intermediate filaments, and adhesive structures on cells as they respond to various geometric configurations, including pillars, columns and spikes, has been analyzed on microfabricated substrates [179, 189].

2.2.2.2 Biochemical Modifications: Creating an “Active” Nerve Conduit

Eliciting a desirable reaction from the host tissue after nerve injury has a profound influence on the regenerative capacity of the nervous tissue. It has been determined that the response of the host tissue is related to not only the mechanical and physical properties of the implanted biomaterial but the chemical properties also play a strong role in promoting a beneficial response from the native environment [143]. Manipulating the natural repair process of the nervous system by engineering a
specific biochemical response from the matrix within the conduit or through delivery of growth and neurotrophic factors is an attractive strategy for enhanced nerve regeneration.

2.2.2.2.1 Chemical Patterning

Providing an adhesive substrate for cellular and neurite growth is an important mechanism for guidance. To control cell adhesion, migration as well as tissue growth and repair, scaffolds for neural tissue engineering incorporate specific bioactive chemicals. Several studies have been performed using different methods for generating patterns of adhesive domains on various materials. These studies have largely consisted of two-dimensional substrates where adhesive areas are patterned adjacent to nonadhesive areas. However, chemical patterning of three-dimensional substrates has also been demonstrated. The techniques developed for these in vitro studies are then applied to create precise patterns of adhesive domains in conduits [190] to be used in in vivo experimentation. Patterning techniques manipulating surface chemistry and using photolithographic photomasks have aided in the reproducible creation of desired patterns of biological molecules on surfaces. Microstamping hexadecanethiol on gold in a self-assembled monolayer (SAM) has been used to create islands of various shapes that support the adsorption of many proteins [178]. Similar methods have been used to print poly-L-lysine, laminin and bovine serum albumin directly on surfaces using texturized silicone stamps dipped in protein, dried, and transferred onto chemically modified substrates [191, 192]. Microfabrication techniques have also been used in the photolithographic patterning of organosilanes to silicon wafers [181, 182], quartz [184] and standard glass [188, 193]. Several chemicals and proteins have been employed to create regions of two-dimensional patterns of adhesive domains: laminin [194]; nerve growth factor [195]; fibronectin; collagen; albumin; and laminin paired with other chemicals including laminin-denatured laminin, laminin-albumin, polylysine-conjugated laminin [196] and laminin-collagen [197]. Results from cell experimentation have suggested that biochemical patterning might play a stronger role in inducing cellular response
(i.e. attachment, spreading, and alignment) than topography. Therefore, efforts have been made to combine multiple guidance cues by chemically patterning three-dimensional substrates [153, 175, 176].

2.2.2.2 Matrices within Polymer Conduits

Neural tissue engineering applications focus on mimicking the nerve and the supporting extracellular matrix (ECM) in order to repair or regenerate axons following damage or disease. Experimentation with ECM molecules [198] and the tailoring of matrices within conduits has led to support of axonal regrowth following injury. Evidence has been presented that the basement membrane protein laminin provides pathways of adhesiveness in both peripheral and central nervous system tissues [199]. This ECM protein is capable of initiating and supporting neurite extension on glass and biodegradable polymers of poly (lactide-co-glycolide) and poly(DL-lactide) [174, 193] as well as glial cell outgrowth on polystyrene [153]. Agarose gels derivatized with laminin oligopeptides have enabled three-dimensional neurite outgrowth in vitro from cells containing receptors to the laminin peptides. [200]. These gel matrices provide support, create an environment that supports growth and incorporate materials that alter surface area. Collagen, fibronectin, and fibrin have also been used to enhance cell-substrate interaction [160-162, 201-205]. Gels using magnetic fields have been shown to orient fibers of collagen within the gels. Compared to randomly oriented fibers, these gels promoted neurite extension both in vitro and in vivo [161]. Magnetically aligned fibrin gels (MAFGs) having different fibril diameters but similar alignment (Figure 2.9) resulted in drastic changes in the contact guidance response of neurites. In gels formed in 1.2 mM Ca$^{2+}$ and having a smaller fibril diameter, there was no response from chick dorsal root ganglia. However, a strong response in gels formed in 12 and 30 mM Ca$^{2+}$ with a larger fibril diameter enhanced neurite length two-fold [205]. Furthermore, nerve conduits can be filled with specific bioactive molecules to elicit new axonal growth following injury. Such molecules including axon guidance and pathfinding
molecules (netrins, semaphorins, ephrins, Slits) [206], cell adhesion molecules (CAMs) that promote neurite growth (NCAM, L1, N-cadherin, tenascin) [207, 208], as well as proteins involved in synaptic differentiation (agrin, laminin beta 2, and ARIA) [209] have numerous applications for nerve regeneration in vivo.

Synthetic hydrogels have served as artificial matrices for neural tissue reconstruction, for the delivery of cells and for the promotion of axonal regeneration required for successful neurotransplantation. Cultured neurons were found to attach to hydrogel substrates prepared from poly (2-hydroxyethylmethacrylate) (PHEMA) but grow few nerve fibers unless fibronectin, collagen, or nerve growth factor was incorporated into the hydrogel. This provides a mechanism to provide controlled growth on hydrogel surfaces [210]. Hydrogels have been created with bioactive characteristics for neural cell adhesion and growth [211]. Arg-Gly-Asp (RGD) peptides were synthesized and chemically coupled to the bulk of poly (N-(2-hydroxypropyl) methacrylamide) (PHPMA) based polymer hydrogels. These RGD-grafted polymers implanted into the striata of rat
brains promoted and supported the growth and spread of glial tissue onto and into the hydrogels [212]. Cultured Schwann cells, neonatal astrocytes or cells dissociated from embryonic cerebral hemispheres were also dispersed within PHPMA hydrogel matrices and found to promote cellular ingrowth in vivo [213]. These polymer hydrogel matrices were found to have neuroinductive and neuroconductive properties and the potential to repair tissue defects in the central nervous system by promoting the formation of a tissue matrix and axonal growth by replacing lost tissue [152, 214-216]. Furthermore, in the injured adult and developing rat spinal cord, these biocompatible porous hydrogels (NeuroGels) promoted axonal growth within the hydrogels, and supraspinal axons migrated into the reconstructed cord segment [217].

### 2.2.2.3 Neurotrophins

Neurotrophins are proteins in the nervous system that regulate neuronal survival and outgrowth, synaptic connectivity and neurotransmission. These growth-promoting factors are used to functionalize guidance conduits and create a desired response from the regenerating neural environment. Manipulation of polymer conduits for growth factor administration may be a useful treatment for neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease, which are characterized by the degeneration of neuronal cell populations. There is also much potential for overcoming severe tissue loss using growth factors released from nerve conduits in cases where there is a large gap as a result of axotomy. Various neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) as well as other important growth factors and cytokines, including ciliary neurotrophic factor (CNTF), glial cell line-derived growth factor (GDNF), and acidic and basic fibroblast growth factor (aFGF, bFGF), have been “trapped” inside polymer conduits that control their release.

Nerve growth factor (NGF) was one of the earliest neurotrophic factors identified and is one of the most thoroughly studied neurotrophins. In early experiments incorporating NGF into silicone
chambers, the effects of NGF on nerve regeneration were positive but limited. This was attributed to the rapid decline in NGF concentrations in the conduit due to degradation in aqueous media and leakage from the conduit [218]. The method of delivery of these factors is a challenge and such limitations were overcome by providing controlled release of NGF. Controlled-release polymer delivery systems may be an important technology in enabling the prevention of neuronal degeneration, or even the stimulation of neuronal regeneration, by providing a sustained release of growth factors to promote the long-term survival of endogenous or transplanted cells [219].

Polymeric implants providing controlled release of NGF for one month were developed and found to improve neurite extension in cultured PC12 cells [220]. Continuous delivery of NGF has been shown to increase regeneration in both the PNS [221, 222] and the CNS [223, 224]. Furthermore, in an effort to readily provide for prolonged, site-specific delivery of NGF to the tissue, without adverse effects on the conduit, biodegradable polymer microspheres of poly (L-lactide) co-glycolide containing NGF were fabricated. Biologically active NGF was released from the microspheres, as assayed by neurite outgrowth in a dorsal root ganglion tissue culture system [225-227]. NGF co-encapsulated in PLGA microspheres along with ovalbumin was found to be bioactive for over 90 days [228]. Sustained release of NGF within nerve guide conduits has also been tested. NGF release from biodegradable poly(phosphoester) microspheres produced using a double emulsion technique exhibited a lower burst effect but similar protein entrapment levels and efficiencies when compared with those made of PLGA [229]. These NGF-loaded poly(phosphoester) microspheres were successfully implanted to bridge a 10 mm gap in a rat sciatic nerve model. Furthermore, the exogenous NGF had long-term morphological regeneration effects in the sciatic nerve [230].

Basic fibroblast growth factor (b-FGF) has been shown to enhance the in vitro survival and neurite extension of various types of neurons. One of the earliest studies involved controlled release of b-FGF and alpha-1 glycoprotein (α1-GP) from synthetic nerve guidance channels fabricated using the dip molding technique. After an initial burst in the first day, linear release was obtained from the
conduits for a period of at least two weeks afterward. Only the tubes releasing b-FGF or b-FGF and alpha 1-GP displayed regenerated cables bridging both nerve stumps, which contained nerve fascicles with myelinated and unmyelinated axons [231]. Biodegradable polymer foams modified with a-FGF and used for controlled release and the provision of a permissive environment for spinal cord regeneration were formed using freeze-drying techniques [232]. Furthermore, evidence has been shown that a-FGF and b-FGF promote angiogenesis and may aid in the repair of damaged nerves [233].

Other neurotrophic factors such as GDNF, BDNF and NT-3 have been released from synthetic guidance channels. In an effort to study facial nerve regeneration, the effects of the cytokine growth factor, GDNF, and NT-3 on nerve regeneration were assessed after rat facial nerve axotomy. Nerve cables regenerated in the presence of GDNF showed a large number of myelinated axons while no regenerated axons were observed in the absence of growth factors, demonstrating that GDNF, as previously described for the sciatic nerve, a mixed sensory and motor nerve, is also very efficient in promoting regeneration of the facial nerve, an essentially pure motor nerve [234]. Exogenous BDNF and NT-3 were delivered simultaneously into Schwann cell-grafted semipermeable guidance channels by an Alzet minipump to test the ability of these neurotrophins to promote axonal regeneration. This novel experiment elegantly demonstrated that BDNF and NT-3 infusion enhanced propriospinal axonal regeneration and enhanced axonal regeneration of specific distant populations of brain stem neurons into grafts in the adult rat spinal cord [235].

2.2.2.3 Cellular modifications

Due to certain limitations with the control of growth factor delivery systems, cells have been manipulated for the direct delivery of certain neurotrophic factors using a variety of therapeutic strategies. Genetic engineering has been used to modify cells for neurotrophic factor delivery [236]. Cells that produce specific neurotrophic and growth factors have been encapsulated using polymeric
biomaterials. Other experiments have involved the direct seeding of cells known to secrete neurotrophic factors, such as Schwann cells, olfactory ensheathing cells (OECs) and neural stem cells (NSCs), into nerve conduits.

2.2.2.3.1 Cell Encapsulation

Implanting polymer-encapsulated cells for secreting growth and neurotrophic factors has been used for treatment for neurodegenerative disorders and to promote nerve regeneration. As an experimental therapy for Parkinsonian patients, enhanced benefit from neural transplantation can be provided through the combination of grafting with trophic factor treatment. This strategy ultimately results in improved survival and growth of grafted embryonic dopaminergic neurons. It has been demonstrated that the implantation of polymer-encapsulated cells genetically engineered to continuously secrete GDNF to the adult rat striatum improves dopaminergic graft survival and function. This shows that polymer encapsulation of cells can be used as an effective vehicle for long-term trophic factor supply [237]. A number of proteins have specific neuroprotective activities in vitro; however, the local delivery of these factors into the central nervous system over the long term at therapeutic levels has been difficult to achieve. Direct administration at the target site is a logical alternative, particularly in the central nervous system, but the limits of direct administration have not been defined clearly. For instance NGF must be delivered within several millimeters of the target to be effective in treating Alzheimer's disease [238]. Cells engineered to express neuroprotective proteins, encapsulated in immunoisolation polymeric devices and implanted at the site of lesions have the potential to alter the progression of neurodegenerative disorders. The polymers used for encapsulation should allow transport of nutrients and oxygen to the cells, but also afford immunoprotection. Long-term cell viability in vivo in these constructs due to diffusional limitations has been the major drawback of this approach.
Ciliary neurotrophic factor (CNTF) decreases naturally occurring and axotomy-induced cell death and has been evaluated as a treatment for neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and Huntington’s disease [239]. Effective administration of this protein to motoneurons has been hampered by the exceedingly short half-life of CNTF, and the inability to deliver effective concentration into the central nervous system after systemic administration in vivo. BHK cells stably transfected with a plasmid construct containing the gene for human or mouse CNTF were encapsulated in polymer fibers and found to continuously release CNTF and slow down motoneuron degeneration following axotomy [240]. Therefore cell encapsulation is a potentially important method in nerve regeneration, and can be used alone or in conjunction with other methods such as entubulization.

2.2.2.3.2 Cell Implantation

2.2.2.3.2.1 Schwann cells

Schwann cells play an important role in supporting axonal regeneration after damage or disease. These cells clean debris from the injury site and secrete regulatory proteins that aid in neuronal survival and axonal growth. In the PNS and CNS, Schwann cells organize the regenerating environment through the myelination of axons, production of ECM, CAMs, and neurotrophins as well as aiding in the guidance of regenerating axons. The use of PNS grafts in the CNS by Aguayo et al. in the early 1980’s distinguished Schwann cells as essential for CNS repair [124]. Conduits incorporating these cells have enhanced PNS regeneration [241]. CNS regeneration has also been induced after implantation of the semipermeable polyacrylonitrile/polyvinylchloride (PAN/PVC) and Matrigel conduits seeded with Schwann cells into the transected rat spinal cord [131, 242]. Poly (α-hydroxy acids) with seeded Schwann cells or Schwann cell grafts were also found to be effective candidates for spinal cord regeneration [129, 138]. However, limited regeneration has been demonstrated between the implant and the distal end of the host spinal cord [136] and myelination
beyond the injury site has not been observed [242]. Research applicable to human applications has demonstrated that implantation of human Schwann cells in the nude (T cell deficient) rat spinal cord allowed axonal growth across the graft and re-entry into the spinal cord [243].

2.2.2.3.2.2 Olfactory Ensheathing Cells

Olfactory bulb ensheathing cells (OECs) are the primary glial cells found in both the PNS and CNS of the olfactory system. This system differs from other CNS tissues in that axons continue to grow throughout adulthood. These cells share characteristics with astrocytes of the CNS and Schwann cells from the PNS. Like astrocytes, these cells express glial fibrillary acidic protein (GFAP), yet they ensheath and myelinate axons and support axonal regrowth, which are features of Schwann cells. Most work in this area has involved OEC transplantations performed to promote remyelination of demyelinated rat spinal cord axons [244, 245] and foster regeneration of damaged axons in the mature CNS [132, 246-248]. Incorporating OECs into conduits has promoted axonal regeneration in both the PNS [249] and CNS [247] using Schwann cell-filled guidance channels. The results from these transplantations have demonstrated a distinct advantage of these cells over Schwann cells in creating a regenerative environment within the CNS.

2.2.2.3.2.3 Neural Stem Cells

Neural stem cells (NSCs) were seeded into a dual scaffold structure made of biodegradable polymers to address the issues of spinal cord injury. Unique biodegradable polymer scaffolds were fabricated where the general design of the scaffold was derived from the structure of the spinal cord with an outer section that mimics the white matter with long axial pores to provide axonal guidance and an inner section seeded with neural stem cells for cell replacement and mimic the general character of the gray matter [250] (Figure 2.10). The seeded scaffold improved functional recovery as compared with the lesion control or cells alone following spinal cord injury. Implantation of the
scaffold-neural stem cells unit into an adult rat hemisection model of spinal cord injury promoted long-term improvement in function that was persistent up to one year in some animals, relative to a lesion-control group [127].

**Figure 2.10:** (a) Schematic of the scaffold design showing the inner and outer scaffolds. (b and c) Inner scaffolds seeded with NSCs. (Scale bars: 200 μm and 50 μm, respectively.) The outer section of the scaffold was created by means of a solid–liquid phase separation technique that produced long, axially oriented pores for axonal guidance as well as radial pores to allow fluid transport and inhibit the ingrowth of scar tissue (d; scale bar, 100 μm). (e) Schematic of surgical insertion of the implant into the spinal cord [127].

Human embryonic stem (hES) cells hold promise as an unlimited source of cells for transplantation therapies [251]. However, control of their proliferation and differentiation into complex, viable 3D tissues is challenging. Combining physical support with chemical cues created a supportive environment for the control of differentiation and organization of hES cells. Langer *et al.* developed biodegradable poly (lactic-co-glycolic acid)/poly (L-lactic acid) polymer scaffolds to promote hES cell growth and differentiation and formation of 3D structures. Complex structures with features of various committed embryonic tissues were generated *in vitro* using early differentiating hES cells and using the supportive three-dimensional environment to further induce differentiation. Growth factors such as retinoic acid, transforming growth factor β, activin-A, or insulin-like growth
factor directed hES cell differentiation and organization within the scaffold resulting in the formation of structures with characteristics of developing neural tissues, cartilage, or liver as well as the formation of a 3D vessel-like network. These constructs were transplanted into severe combined immunodeficient mice and continued to express specific human proteins in defined differentiated structures. This recent study presents a novel mechanism for creating viable human tissue structures for therapeutic applications [133].

2.3 Summary

Engineering regeneration in the nervous system presents many challenges. Many strategies that may enhance regeneration in the PNS cannot be applied directly to the CNS due to the complexity of the environment. Novel tissue engineering strategies and mechanisms, including the use of three dimensional polymer constructs with or without biological components (i.e. cells) and products fabricated for the induction of specific responses (i.e. regeneration), and the manipulation of biological cells in vitro (i.e. stem cells or cells for neuronal support), hold much promise for the enhancement of functional repair and replacement of tissue function. The successful use of polymeric nerve conduits in facilitating peripheral nerve regeneration has been demonstrated and polymers have shown great promise in addressing spinal cord injuries as well. This regeneration process, with various polymers, both degradable as well as non-degradable, has been enhanced further by promoting directed growth and by the addition of chemical cues such as ECM molecules, nerve growth factors and neurotrophins and other agents incorporated in the conduits to be released in a controlled fashion. Polymers have also played an important role in encapsulating cells and in the transplantation of neuronal support cells that release factors to promote nerve regeneration. Multidisciplinary tissue engineering approaches involving such biomaterials to mimic the native neural environment of the body have resulted in significant progress in gaining some understanding of the systems and signals involved in nerve regeneration. Incorporating multiple guidance cues and
experimental strategies will allow further opportunities to elucidate the mechanisms behind nerve
regeneration and specific considerations for the efficient repair of the nervous system.

Combining chemical patterning techniques with other guidance mechanisms involving
physical and biological modifications leads to the creation of permissive substrates for \textit{in vitro}
experimentation into the regenerative potential of the optic nerve, and ultimately, the central nervous
system as a whole. Using such strategies, we are transferring patterns of micron scale dimensions
onto synthetic polymers, chemically modifying them with laminin and integrating the biological
influence of astrocytes to explore the growth, differentiation and alignment of adult hippocampal
progenitor cells (AHPCs) on micropatterned polystyrene substrates.
2.4 References


CHAPTER 3

ORIENTED ASTROGLIAL CELL GROWTH ON MICROPATTERNED POLYSYRENE SUBSTRATES

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3.1 Abstract

In an effort to develop a permissive environment for neural stem cell differentiation, directional growth of astrocytes has been achieved on polymer substrates *in vitro*. Manipulating a combination of physical and chemical cues, astrocyte adhesion and alignment *in vitro* were examined. To provide physical guidance, micropatterned polymer substrates of polystyrene (PS) were fabricated. Laminin was selectively adsorbed onto the grooves of the patterned surface. Rat type-1 astrocytes were seeded onto the micropatterned PS substrates, and the effects of substrate topography and the adsorption of laminin to the PS substrates on the behavior and morphology of the astrocytes were explored. The astrocytes were found to align parallel to the micropatterned grooves at initial seeding densities of approximately 7500, 13,000, and 20,000 cells per cm² due to the effects of the physical and chemical guidance mechanisms. Adsorbing laminin in the microgrooves of the micropatterned PS substrates improved cell adhesion and spreading of cytoskeletal filaments significantly. At these initial seeding densities, over 85% astrocyte alignment in the direction of the grooves was achieved.

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on the micropatterned PS substrates with laminin adsorbed in the grooves. This combination of guidance cues has the potential to provide a permissive substrate for \textit{in vivo} regeneration within the central nervous system.

### 3.2 Introduction

Providing a permissive environment for the regeneration of axons is essential to central nervous system (CNS) repair [1, 2]. Astrocytes, neuroglial cells of the CNS, provide trophic and tropic support for the proliferation, survival, and maturation of developing neurons. Astrocytes are involved in metabolic functions of neurons, guide the migration of growing axons during development [3, 4], and regulate neuronal shape and synaptic connectivity [5]. Astrocytes are also capable of integrating neuronal inputs and modulating synaptic activity [6, 7]. Furthermore, recent evidence has shown that mature astrocytes can take an active role in the induction of neurogenesis from adult neural stem cells [8]. Due to the nature of the interactions between astrocytes and neurons and neural stem cells in the CNS, astrocytes can potentially be used as biological cues to provide a permissive environment for the regeneration of CNS axons.

Another key factor in CNS regeneration is directed neuronal outgrowth. \textit{In vivo}, extracellular matrix and oriented tissue structures influence cell migration and axon outgrowth [9]. Directed growth of astrocytes on polymer substrates can potentially promote controlled outgrowth and differentiation of neural stem cells. Cells recognize three-dimensional geometric configurations of substrate surfaces and their growth can be controlled and guided through the fabrication of microgrooves and other patterns on these surfaces [10-14]. Cellular response to the surrounding topography depends on the characteristics of the substrate, including the types, densities and magnitudes of the features, as well as cell type and interactions with neighboring cells [11-13, 15, 16]. Several studies have been performed using techniques that manipulate surface chemistry and
photolithography to generate patterns of cell-adhesive domains on two-dimensional and three-dimensional substrates of various materials [17-22]. Using such techniques, guided outgrowth of Schwann cells and dorsal root ganglia were demonstrated in our laboratory using micropatterned polymer surfaces [15, 16].

Integrating the biological influence of astrocytes and physical and chemical guidance cues potentially creates a permissive environment for the selective differentiation and outgrowth of neural stem cells and is a promising avenue for control of repair and regeneration in the injured or diseased CNS. Physical and chemical mechanisms that direct and guide astrocyte adhesion and alignment *in vitro* were investigated as a first step in this process. This paper focuses on the fabrication of micropatterned polymer surfaces chemically modified with adhesive proteins and the investigation of the influence of physical and chemical guidance cues on the behavior and morphology of astrocytes growing on these micropatterned substrates *in vitro*.

3.3 Materials and Methods

3.3.1 Micropatterned substrate fabrication

Polystyrene (PS) was chosen for substrate fabrication, as it is a biocompatible polymer that is used extensively in cell culture experimentation. Conventional photolithographic techniques were used to prepare silicon wafers with the desired micropatterns that were then transferred to the polymer substrates using solvent casting. The patterns used were described by: groove width (μm)/ groove spacing (or mesa width) (μm)/ groove depth (μm). Pattern optimization was performed by our group in the recent past using dorsal root ganglia and Schwann cells [15, 16, 23]. Substrates with patterns of 10/10 μm and 10/20 μm were found to provide the best Schwann cell and neurite alignment [15, 16, 23]. Groove depths greater than 2 μm acted as physical barriers, which enabled better alignment of the cells [16, 23]. To study the physical guidance of astrocytes on micropatterned PS substrates, the pattern dimensions used for these experiments were 10/20/3 μm.
These dimensions were used with the hypothesis that astrocytes will exhibit behavior similar to Schwann cells and dorsal root ganglia.

A chrome lithographic photomask with the desired geometric patterns was used to selectively expose regions of four-inch diameter silicon wafers. These wafers were coated first with 1 kÅ of chrome under an ultra-high vacuum at less than 1μTorr (Figure 1 - Step 1) and then with a positive photoresist (AZ 5209E, Clariant, Somerville, NJ) (Figure 1 - Step 2). After prebaking at 100°C for 1 minute on a hotplate, the photoresist was exposed to ultra-violet radiation through a patterned chrome photomask (Figure 1 - Step 3). Exposed photoresist was removed using AZ 312 MIF developer (Clariant) for 60 seconds to etch the exposed photoresist (Figure 1 - Steps 4 and 5). After post-baking at 120°C for 25 minutes, the exposed chrome was removed with CR4 commercial chrome etch (82% water, 9% nitric acid and 9% ceric ammonium nitrate) (Figure 1 - Steps 6, 7, and 8), and the photoresist was removed by flowing acetone over the silicon wafers (Figure 1 - Step 9). After patterning the metal with the desired dimensions, the silicon wafer was etched in the Reactive Ion Etcher (Plasma-Therm 720, dual-chamber, Leybold 360 turbo-pump, St. Petersburg, FL) at a rate of 16.8 nm per minute and the chrome covering the mesas was removed. Silicon wafers used as microdies for solvent cast substrates were also created using the Deep Reactive Ion Etcher (DRIE) (Alcatel 601E Micromachining Etch System, Annecy, France) to create deeper and more anisotropic grooves at a faster etch rate. The processing of the wafers before deep reactive ion etching involved similar steps to those mentioned above for the RIE with exception of metal deposition. Due to the relatively short etch times for DRIE as compared to the RIE, only patterned photoresist was necessary to use as a mask while the silicon wafer was being etched in the DRIE.

The solvent cast polymer substrates were fabricated from a 7-10% (w/v) polystyrene (PS) (MW 125,000-250,000) (Polysciences, Inc., Warrington, PA) solution in toluene. The micropatterned PS substrates were created by spin casting or gravity casting the polymer solution onto the etched silicon wafer with the desired pattern. Substrate thicknesses of approximately 50-70 μm were
achieved using solvent casting techniques. After casting and drying for a minimum of 24 hours, the PS substrate was removed by soaking in deionized (DI) water and then sterilized with 70% ethanol.

The micropatterned substrates were imaged using scanning electron microscopy (SEM). After mounting, the samples were sputter coated (SEM Coating Unit E5100, Polaron Instruments, Inc., Watford Hertfordshire, UK) with 15 nm of gold. The SEM images were taken using a JEOL JSM-840A at an accelerating voltage of 20 kV, a 50 μm diameter aperture and a vacuum level of 1 x 10^-6 Torr.

3.3.2 Growth chambers for cell seeding

Cell growth chambers were constructed using PTFE (Teflon®) o-rings (Small Parts, Inc., Miami Lakes, FL) with inner diameters of 9/16 inches, outer diameters of 3/4 inches and widths of 3/32 inches, glass coverslips, and the PS substrates. The glass coverslips (22 mm by 22 mm, Corning, Corning, NY) and o-rings were sterilized in 70% ethanol, and the o-rings were autoclaved following ethanol sterilization. The o-rings were then attached to the coverslips using Syl-Gard® (Dow Corning Corp., Midland, MI). The PS substrates were cut into squares approximately 1 cm² in area with 0.5 cm² of patterned substrate adjacent to 0.5 cm² of non-patterned substrate (used as a control) (Figure 2). The PS substrates were secured to the glass coverslips within the inner diameter of the o-ring using Silastic® medical adhesive (Dow Corning Corp.) resulting in a growth chamber for the cells. Each chamber was then placed inside a sterile, 35 mm petri dish (Falcon, Franklin Lakes, NJ) (Figure 2). Laminin was applied to the PS substrate within this chamber prior to the seeding of the cells (see below).

3.3.3 Laminin adsorption onto polystyrene substrates

Laminin (Sigma, St. Louis, MO) was prepared in Earle’s Balanced Salt Solution (EBSS; Gibco, Grand Island, NY) at a concentration of 0.01 mg/ml. Laminin was selectively incorporated in
the microgrooves using a surface tension based technique [15]. Approximately 0.5 ml of laminin solution was placed onto the polystyrene substrates (within the Teflon o-ring growth chambers), allowed to adsorb for 15 minutes and removed with a micropipette tip placed perpendicular to the surface of the substrate. The excess solution was allowed to dry on the surface for at least 4 hours at room temperature before seeding cells onto the PS substrates.

To observe the distribution of laminin on the surface of the micropatterned substrate, fluorescein isothiocyanate (FITC; Fisher Scientific, Pittsburgh, PA) was conjugated to laminin using protein labeling techniques. Prior to labeling, laminin at 1 mg/ml in Tris-HCl with 150 mM NaCl, pH=7.5, was exchanged into borate buffer (310 mg H₃BO₃, 475 mg Na₂B₄O₇•10 H₂O in 100 ml ddH₂O, pH 8.4) by dialysis using a Slide-A-Lyzer Dialysis Unit (Pierce Biotechnology, Rockford, IL) with a molecular weight cut-off (MWCO) of 10,000. Laminin was then reacted with 2 µl of FITC at 10 mg/ml in DMSO (Sigma), incubated for one hour and then dialyzed in phosphate buffered saline (PBS) using a 10,000 MWCO Slide-A-Lyzer Dialysis Unit. The FITC-conjugated laminin was then applied to the patterned PS substrates and visualized with fluorescence microscopy using a FITC filter having a 495 nm absorption cutoff and confocal microscopy (Prairie Technologies, Madison, WI) using an 488 nm Argon laser. Confocal images 0.5 µm apart were taken through each substrate analyzed and used to reconstruct a three-dimensional representation of the sample. All image analysis was done using MetaMorph software (Universal Imaging Corporation, West Chester, PA).

3.3.4 Astroglial cell isolation and purification

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. A population of purified astrocytes was obtained from the cerebral cortex of neonatal rat pups. The methods described below were modified from Innocenti and co-workers [24] and Parpura and co-workers [25]. Strategies used include the selection of nervous tissue where neurogenesis is completed but immature astroglial cells are present as the major
proliferative cell population. Vigorous mechanical tissue dissociation procedures were used that destroy the majority of neurons but allow the survival of small undifferentiated glial precursor cells. Briefly, cerebral hemispheres were freshly dissected from 1-3 day old Sprague-Dawley rat pups. The meninges were removed from each cerebral hemisphere, the hemispheres were washed in EBSS and then enzymatically treated with papain solution (20 IU/ml; 37 °C, 5% CO₂/95% air, 1 hour) (Sigma). After subsequent treatment with trypsin inhibitor solution (10 mg/ml; Sigma), the tissue was mechanically dissociated in modified minimal essential culture medium (MMEM). The cultures were grown to confluence in 25 cm² tissue culture flasks (T-25; Falcon) at 37 °C in a humidified 5% CO₂/95% air atmosphere. The culture medium, MEM, consisted of minimum essential medium (MEM; Gibco) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate and 14 mM NaHCO₃, penicillin (100 IU/ml) and streptomycin (100 µg/ml) with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT), pH 7.35.

After the cultures reached confluency (~8 days), an enriched population of type-1 astrocyte cultures was prepared by taking steps to eliminate both neurons and microglia from the flask. Neurons do not survive after washing with cold MEM. Microglia were jarred loose from the astrocyte bed by shaking the flasks. After ~8 days, the cells were washed with cold medium two times and shaken twice on a horizontal shaker at 260 RPM at 37 °C, first for 1.5 hours and then after replacement of the medium, again for 18 hours. The remaining adherent cells were enzymatically detached with trypsin (0.1% in EBSS; Sigma). Cells were then pelleted (100 X g, 10 min.), resuspended in MEM and passaged into 25 cm² tissue culture flasks. The astrocytes attached to the flask within 18 hours. Cultures were fed every 3 days. Cells were not passaged more than 8 times.

3.3.5 Astroglial cell seeding onto micropatterned substrates

After rinsing with EBSS, the cells were detached from the culture flasks using trypsin (0.1% in EBSS; 5 minutes), pelleted (100 X g, 10 min) and resuspended in the appropriate volume of
medium or EBSS for live staining of the astrocytes prior to cell counting. The astrocytes were stained in cell suspension with carboxyfluorescein diacetate succinimidyl ester (CFDA SE; Molecular Probes, Eugene, OR) (5 μM), having an absorption wavelength of 495 nm and an emission maximum of 525 nm. A viable cell count for the astrocytes was determined by trypan blue cell exclusion using a hemacytometer. Astrocytes were seeded onto micropatterned/non-patterned substrate surfaces coated with laminin (0.01 mg/ml EBSS) and those not coated with laminin at initial densities varying from 7,500 to 20,000 cells/cm² and incubated at 37 °C in a 5% CO₂/95% air atmosphere. Higher seeding densities were also seeded onto the micropatterned substrates but were not analyzed due to difficulty with measuring individual astrocyte orientations. Such seeding densities resulted in a monolayer of cells spanning the grooves for which individual cells could not be analyzed accurately.

The astrocytes were observed using light microscopy (Olympus IMT-2 bright field/phase contrast microscope) and epifluorescence microscopy (Nikon Corp., Melville, NY) during culture. Digital images were taken throughout experimentation using the epifluorescence microscope (Nikon Corp.) equipped with a cooled digital camera (ORCA, Hamamatsu) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA). Cultured astrocytes were also examined using a photomicroscope (Microphot FXA; Nikon Corp.). Images were captured from the photomicroscope with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a framegrabber (Megagrabber; Perceptics, Knoxville, TN), in a Macintosh 8100/80AV computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

3.3.6 Immunocytochemistry and histological staining

Histological and immunocytochemical staining procedures were used to enhance the visibility of the astrocytes on the PS substrates as well as determine if the cultures used for experimentation were type-1 astrocytes that were free of neurons and other types of glial cells. To
observe the astrocytes as they interacted with the PS substrates, live cell staining procedures involving the tracer mentioned previously, CFDA SE, were used. At 24 or 72 hours after seeding the astrocytes onto the substrates, astrocytes were fixed using 4% paraformaldehyde in 0.1 mM PO₄ buffer. Following fixation, histological and immunocytochemical techniques were performed. When used in combination, the immunocytochemical procedures preceded the histological staining of the astrocytes.

Indirect immunofluorescence was performed on cells cultured on glass coverslips (12 mm round; Fisher Scientific) coated with 1 mg/ml poly-L-lysine (Sigma) prepared in borate buffer at 72 hours after seeding and on the PS substrates at 24 or 72 hours after seeding. Immunocytochemistry was performed using monoclonal antibodies directed against glial fibrillary acidic protein (GFAP; Anti-GFAP, G-A-5, mouse IgG) purchased from ICN Immunobiologicals, Inc. (Costa Mesa, CA) and diluted 1:200, and polyclonal anti-GFAP antibody (rabbit IgG, Sigma) diluted 1:100. These antibodies were used as markers for astrocytes, as GFAP is a type of intermediate filament found in astrocytes. Neurons were identified using an antibody directed against microtubule associated protein 2 (MAP-2ab; 1:150), purchased from Sigma (mouse IgG). Terminally differentiated oligodendrocytes were identified using Anti-Rip (1:50), obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biology, University of Iowa, under contract NO1-HD-2-3144 from the NICHD (mouse IgG). Goat anti-mouse IgG secondary antibodies conjugated with Alexa 546 (diluted 1:100) or Alexa 488 (1:150) were purchased from Southern Biotechnology (Birmingham, AL) or Molecular Probes (Eugene, OR). Goat anti-rabbit IgG secondary antibody conjugated with RITC (diluted 1:150) was purchased from Southern Biotechnology (Birmingham, AL). All primary and secondary antibodies were diluted in blocking solution (5% goat serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% TritonX-100 (Fisher Scientific)). Negative controls were performed in parallel by omission of the primary and secondary antibodies. No antibody labeling was observed in the controls.
Following fixation alone or fixation and immunocytochemistry, the F-actin cytoskeleton was labeled with Alexa Fluor 568 phalloidin (Molecular Probes) diluted at 1:40 in PBS. Nuclei of the astrocytes were stained with 4', 6- diamidino-2-phenylindole, dilactate (DAPI; Molecular Probes), diluted at 1:100 in PBS. Following labeling with phalloidin and then DAPI, the preparations were mounted onto microscope slides using an antifade mounting media (Gel Mount; Biomek Corp., Foster City, CA).

3.3.7 Assessment of GFAP immunoreactivity

Following immunocytochemical procedures involving GFAP on the coverslips and PS substrates, the preparations were examined on a photomicroscope (Microphot FXA; Nikon Corp.). A 20X objective was used to examine 10 to 20 microscope fields, each field representing 0.1 mm² (360 μm by 280 μm). The following counts were made in each microscope field for the coverslips and the PS substrates: the total number of cells (using a DAPI filter cube) and the number of cells expressing the primary antibody of interest (using a TRITC and FITC filter cube, respectively). This data was used to calculate the percentage of cells labeled with one of the antibody markers (mentioned above) on each coverslip and anti-GFAP alone on the PS substrates. For PS substrates, the data collected after 24 and 72 hours was compared and analyzed.

3.3.8 Determination of cell alignment

At 24 and 72 hours after seeding, the cells were fixed, labeled and mounted onto glass microscope slides (Fisher Scientific, Pittsburgh, PA). The cells were examined and photographed using fluorescence microscopy (Nikon epifluorescence microscope with Hamamatsu digital camera) with a 20X objective, each field representing approximately 0.24 mm² (546 μm by 438 μm). The effect of initial cell seeding density and the laminin adsorbed on the substrate was evaluated for the pattern dimensions 10/20/3 μm. The orientation of the astrocytes on the PS substrates was measured
quantitatively using MetaMorph software (Universal Imaging Corp., West Chester, PA) as the angle of the longest chord through each astrocyte relative to the horizontal axis of the imported image. The data were grouped in 10° sectors between -90° to 90°. Orientations of the groove position in the images were measured in the same way. Control data were taken from measurements made on astrocytes on non-patterned PS substrate areas adjacent to the patterned areas. The angle of orientation in these controls was measured relative to the horizontal axis (0°). Statistical analysis was performed on the values of the differences between the orientation of the cell and the orientation of the groove on the substrate with a difference of 0° indicating perfect alignment.

Cell alignment was measured as the proportion of astrocytes whose longest chord makes an angle of ≤ 20° with the direction of the grooves in these studies. The proportions of cells falling in this group as well as those astrocytes whose longest chord made an angle of ≤ 10° with the direction of the grooves were estimated. There were four types of substrates examined: (1) micropatterned PS substrates with laminin adsorbed on the surface (LAM - PATT); (2) micropatterned PS substrates without laminin (NO LAM - PATT); (3) non-patterned PS substrates with laminin adsorbed on the surface (LAM - NO PATT); and (4) non-patterned PS substrates without laminin (NO LAM - NO PATT). Table 1 displays a summary of the experimental set-up used in analysis of the astrocytes on the micropatterned PS substrates. Three replicates of each treatment or control were analyzed. Statistical analyses were performed on (1) the astrocyte alignment to within 10° and 20° of groove direction and (2) on the number of cells adhering to substrates with laminin as compared to those without. The analysis was performed using the PROC MIXED procedure in SAS statistical software. The data was analyzed using an ANOVA with three error terms to account for (1) the whole plot effect, (2) the split plot effect, and (3) the subsampling due to the experimental design. The whole plot experimental unit was the entire polystyrene substrate (approximately 1 cm² in area) while the split plot experimental unit was the half of the substrate that was either patterned or not patterned. Multiple observations were made on each half of a substrate. The numbers of observations were not
balanced, and this accounted for the subsampling variation. A natural log transformation was used to account for the exponential rate of growth of the astrocytes.

3.4 Results and discussion

3.4.1 Micropatterned substrate fabrication

An efficient system was developed to produce micropatterns having various pattern dimensions on polystyrene (PS) substrates. A microdie was fabricated using photolithography and reactive ion etching and used to transfer the desired pattern onto the polymer substrate with solvent casting techniques. These techniques can be used with many polymers, both non-biodegradable and biodegradable. In Figures 3A and 3B, SEM images of the silicon microdie and micropatterned polystyrene substrates used throughout experimentation are shown. The average thickness of the micropatterned solvent cast polymer substrates was approximately 60 μm.

3.4.2 Laminin distribution assay

Laminin was adsorbed onto the substrates using a surface tension based technique. This protein was used because previous work has demonstrated that laminin promotes cell adhesion and cell spreading [26, 27]. Furthermore, it has been revealed that laminin plays a critical role in regeneration within the CNS [28]. Laminin distribution on the PS substrates was assayed using FITC-conjugated laminin. More laminin was observed lining the walls and base of the groove region than on the mesa surface (Figure 4A). These observations were made initially using fluorescence microscopy and were confirmed using confocal microscopy (Figure 4B). Due to surface tension effects, this method of adsorbing the laminin resulted in more laminin collecting in the grooves than on the mesa surface of the micropatterned substrate. Rinsing with PBS after adsorption resulted in a washing effect on the laminin that was concentrated in the grooves initially, spreading it evenly on the
substrate surface. Hence, the substrates were not rinsed prior to seeding. The control substrates without FITC-conjugated laminin adsorbed to the surface did not exhibit any fluorescence.

3.4.3 Astrocyte seeding and histological staining/ immunocytochemistry

Astrocytes were seeded onto micropatterned/non-patterned substrate surfaces without or with laminin (0.01 mg/ml EBSS) adsorbed on the surface at initial densities varying from 7,500 to 20,000 cells/cm². Histological and immunocytochemical staining procedures were used to enhance the visibility of the astrocytes on the polystyrene substrates. Live cell staining procedures were used to observe astrocyte behavior and morphology as the cells grew on the PS substrates. CFDA SE was found to be very effective in labeling the cells in suspension prior to cell seeding and relabeling after adhesion on the substrates if necessary. This tracer gave a strong green fluorescence signal (using the FITC filter) from the cultured cells for four to five days depending on the proliferation rate of the astrocytes.

Immunocytochemistry was used to determine what percentage of the cells in the cultures were, in fact, type-1 astrocytes. Astrocytes were identified on the basis of the presence of glial fibrillary acidic protein (GFAP) immunoreactivity. Cultures were also screened with MAP-2ab and Rip antibodies for neurons and oligodendrocytes, respectively. It was determined that over 85% of the cells seeded onto coverslips were immunopositive for GFAP and, therefore, were astrocytic in nature. No immunoreactivity was observed with the MAP-2ab and Rip antibodies verifying that the cultures were free of neurons and oligodendrocytes, respectively. Furthermore, for cells seeded on laminin coated PS substrates (squares consisting of 0.5 cm² of patterned substrate adjacent to 0.5 cm² of non-patterned substrate), it was determined that over 90% of the cells on these substrates were immunopositive for GFAP (Figure 5). These percentages verify that the vast majority of cells observed on the PS substrates were astrocytes.
Phalloidin and DAPI were used on the preparations following fixation alone or fixation and immunocytochemistry. F-actin microfilaments were labeled with Alexa Fluor 568 phalloidin. In addition to the GFAP antibodies, this stain enabled examination of the orientation of the filamentous cytoskeleton of the astrocytes on the PS substrates. Nuclei of the astrocytes were stained with DAPI. This stain allowed the visualization of the nuclei on the PS substrates and counting of the total number of cells adhering to the substrate after 24 or 72 hours.

3.4.4 Physical guidance on the micropatterned substrates

A valuable technique for fabricating microgrooves with various pattern sizes and spacings on PS substrates was developed and the effects of the three-dimensional pattern on astrocyte behavior in vitro were studied qualitatively and quantitatively. The behavior and morphology of astrocytes on the patterned substrates were compared to that on non-patterned substrates. On the non-patterned substrates, the astrocytes possessed a flattened, spread morphology. The cells were polygonal in shape and extended processes in a radial fashion displaying no particular orientation. In contrast, on the patterned substrates, the astrocytes were observed as having highly elongated extensions of their filamentous cytoskeleton, both F-actin microfilaments and glial intermediate filaments, in the direction of the grooves. Thus, it was apparent that the behavior of the astrocytes was influenced by the three-dimensional topography of the substrate (Figure 5). The 10/20/3 μm pattern had a significant influence on astrocyte orientation. The 10 μm groove width directed the astrocytes to extend their processes along the inside of the groove or at the boundary between the groove and the mesa. The groove spacing of 20 μm influenced the cells to spread in the direction of the grooves.

Astrocyte orientation on the patterned and non-patterned PS substrates was analyzed and compared. Cell alignment was determined by whether the longest chord through the individual astrocytes made an angle of ≤ 20° with the direction of the grooves. The proportions of cells falling in this group as well as those astrocytes whose longest chord made an angle of ≤ 10° with the direction
of the grooves were calculated. For an initial cell seeding density of approximately 20,000 cells per substrate, the evidence suggested that cell orientation on the 10/20/3 μm patterned substrates was significantly different than on the non-patterned substrates with the same trend observed after 24 and 72 hours. Figure 6 shows the distributions of cell orientation (taken as the difference between the astrocyte orientation and the orientation of the grooves) on the 10/20/3 μm patterned and non-patterned substrates. It can be seen that astrocytes on the control or non-patterned substrates were randomly oriented while the majority of astrocytes on the patterned substrates were aligned in the direction of the grooves. It was estimated that a significantly higher percentage of cells aligned on patterned substrates than non-patterned substrates within 20° and 10° of the direction of the grooves (Figure 7). In an effort to influence adhesion and spreading of cells on the substrate and increase the percentage of cells aligning to the direction of the grooves, chemical modifications to the PS substrate were investigated.

3.4.5 Chemical modification of the PS substrate

To improve cell adhesion and spreading of the astrocytes, laminin (0.01 mg/ml in EBSS) was adsorbed onto the surface of the non-patterned PS substrates. Astrocyte adhesion was estimated by evaluating the total cell count for defined regions on the non-patterned PS substrates. It was observed that laminin improved astrocyte adhesion and spreading for an initial cell seeding density of 20,000 cells/substrate. On LAM-NO PATT substrates, after 24 hours, cell adhesion was significantly improved and approximately 3 times more cells adhered to the PS substrates with laminin than without laminin (Figure 8). To account for the exponential rate of growth of the astrocytes, a natural log transformation was used for the statistical analysis of the cell counts per substrate area. Using this transformation, cell adhesion was significantly improved on LAM-NO PATT substrates after 24 hours and after 72 hours as compared to NO LAM-NO PATT substrates analyzed at the same time periods. However, there was no significant difference in the effect of laminin between the 24 and 72
hour data analyzed. After each time period, approximately 3 times more cells adhered to the PS substrates with laminin than without laminin.

At an initial cell seeding density of 20,000 cells per cm\(^2\), astrocyte orientation was also evaluated. The astrocytes exhibited wide processes spread over the laminin coated substrate surface with similar trends in orientation measurements observed and measured after 24 and 72 hours. Astrocytes adhered to LAM-NO PATT PS substrates and exhibited spreading of their filaments as compared to NO LAM- NO PATT PS substrates where the cells often remained in small clusters with little spreading from the cluster over time. Overall, the astrocytes on NO LAM- NO PATT PS substrates appeared smaller with shorter processes and tended not to spread out on the substrates. On the non-patterned PS substrates with or without laminin, the astrocytes were oriented randomly and were not aligned in any particular direction. The percentages of cells growing at an angle of ≤ 20° on the LAM- NO PATT and NO LAM- NO PATT PS substrates were 20.3% ± 4.8% and 20.3% ± 5.4%, respectively (\(\alpha = 0.05\); standard errors for 6 substrates are reported here). The percentages of cells growing at an angle of ≤ 10° on the LAM- NO PATT PS substrates were 8.3% ± 5% and 10.8% ± 5.6% for NO LAM- NO PATT PS substrates (\(\alpha = 0.05\); standard errors for 6 substrates are reported here). Due to the improvement of astrocyte adhesion to laminin and the alignment due to the microgrooved substrates, the combined effect of laminin on the behavior of astrocytes on the 10/20/3 \(\mu m\) pattern was examined.

### 3.4.6 Effect of chemical and physical guidance

The behavior of the astrocytes on the micropatterned (10/20/3 \(\mu m\)) non-patterned substrate surfaces coated with laminin (0.01 mg/ml EBSS) and those not coated with laminin was analyzed for three seeding densities of approximately 7500, 13,000 and 20,000 cells per cm\(^2\). Using a surface tension based technique, laminin was selectively deposited in the groove regions to promote
alignment of the astrocytes. The astrocytes were exposed to more laminin per area in the grooves than on the surface of the mesas. The synergistic effects of the chemical and physical guidance cues of the laminin coated micropatterned PS substrate were studied in terms of astrocyte adhesion and directed growth.

The effect of laminin on astrocyte adhesion was measured quantitatively on all substrate types. For an initial seeding density of 20,000 cells per cm$^2$, it was determined that adsorbing laminin (0.01 mg/ml in EBSS) onto the surface of the micropatterned (10/20/3 μm) PS substrates resulted in the adhesion of significantly more cells to the substrate after 24 hours than non-coated substrates ($\alpha=0.05$) (Figure 8). There was no significant evidence of a difference between the number of cells adhering to patterned and non-patterned PS substrates ($\alpha=0.05$). Cell adhesion and spreading of the astrocytes were improved on the laminin coated patterned PS substrates without lessening the effect of the physical guidance of the pattern. Improved cell adhesion on laminin coated substrates was expected, as laminin is an ECM protein that has two or more domains that bind to laminin receptors on the surface of cells. It is responsible for many cell-basement membrane interactions including adhesion, migration and proliferation [26, 27]. As seen in Figure 9, astrocytes observed on LAM-PATT substrates were flattened and aligned in the direction of the grooves. On NO LAM-PATT substrates, alignment was still observed, however, due to the astrocytes remaining in tight clusters, a lower proportion of the cells were oriented in the direction of the grooves as compared to astrocyte alignment on LAM-PATT substrates.

The combination of laminin and the three-dimensionality of the grooves provided directional guidance for the astrocytes on the PS substrate (Figure 10). The effect of the combination of chemical and physical guidance cues on astrocyte orientation was evaluated. For an initial seeding density of 20,000 cells per cm$^2$, the micropatterned (10/20/3 μm) PS substrates with laminin (0.01 mg/ml in EBSS) in the grooves were found to have a significant effect on astrocyte alignment with similar trends observed and measured after 24 and 72 hours. The laminin concentrated in the grooves
improved initial cell adhesion and elaboration of astrocyte processes in the direction of the grooves significantly. The distributions of cell orientation (taken as the difference between astrocyte orientation and the orientation of the grooves) for the patterned and non-patterned substrates with adsorbed laminin on the surface are presented in Figure 11. Again, it can be seen that astrocytes on the control or LAM-NO PATT substrates were randomly oriented while the majority of astrocytes on the LAM-PATT substrates were aligned in the direction of the grooves. It was estimated with $\alpha = 0.05$ that a significantly higher percentage of cells aligned on LAM-PATT substrates than on LAM-NO PATT substrates within 10° and 20° of the direction of the grooves (Figure 12).

The effect that the pattern had on directing astrocyte alignment was significantly different for micropatterned PS substrates with adsorbed laminin as compared to those without laminin. From statistical analysis performed on the astrocyte orientation data, it was determined with $\alpha = 0.05$ that the presence of laminin had a significant effect on the alignment of the astrocytes on the patterned substrates. For an initial seeding density of 20,000 cells per cm$^2$, the influence of the laminin coated 10/20/3 μm patterned substrate to direct alignment within 20° and within 10° of the groove direction was compared to that on micropatterned substrates without laminin. A significantly higher percentage of cells aligned in direction of the grooves on LAM-PATT substrates than NO LAM-PATT substrates. The percentage of cells growing at an angle of $\leq 20^\circ$ with the direction of the grooves was 85.5% ± 4.8% for LAM-PATT substrates and 70.3% ± 5.4% for NO LAM-PATT substrates ($\alpha = 0.05$; standard errors for 6 substrates are reported here). The percentage of cells growing at an angle of $\leq 10^\circ$ with the direction of the grooves was 60% ± 5% for LAM-PATT substrates and 49.3% ± 5.6% for NO LAM-PATT substrates ($\alpha = 0.05$; standard errors for 6 substrates are reported here).

In an effort to determine the most effective seeding density for alignment, astrocytes were cultured at initial seeding densities of approximately 7500, 13,000 and 20,000 cells per cm$^2$. PS substrates with laminin adsorbed at 0.01 mg/ml EBSS onto the surface resulted in a significantly
higher proportion of attached cells at all seeding densities tested (Figure 13) with no significant
evidence of a difference between the number of cells adhering to patterned and non-patterned
substrates ($\alpha = 0.05$). Furthermore, the influence of the 10/20/3 $\mu$m pattern to direct alignment within
20° and within 10° of the groove direction was evaluated over all three seeding densities (Figure 14).
The percentages of astrocyte alignment on the PS substrates were not significantly different for the
initial seeding densities examined ($\alpha = 0.05$).

3.5 Conclusions

The combination of physical and chemical guidance cues had a significant effect on the
alignment of the astrocytes on polymer substrates in vitro. Conventional photolithographic techniques
and reactive ion etching were used to prepare silicon wafers having desired micropatterns that were
imprinted onto PS substrates using solvent casting. Using a surface tension based technique, more
laminin was selectively adsorbed to the groove region of the micropatterned substrates. Applying
laminin resulted in significant improvement in astrocyte adhesion and spreading of F-actin
microfilaments and intermediate filaments on the polystyrene substrates in the direction of the
grooves. After 24 and 72 hours, approximately 3 times as many cells adhered to substrates with
adsorbed laminin on the surface than those without laminin. Furthermore, the presence of laminin had
a significant effect on the alignment of the astrocytes on the 10/20/3 $\mu$m patterned PS substrates.
After seeding the astrocytes at the initial densities ranging from 7500 to 20,000 cells/cm$^2$, over 85% 
alignment ($\leq 20^\circ$ with the direction of the grooves) was achieved on the micropatterned PS substrates
with laminin adsorbed on the surface. In this range of initial seeding densities, the astrocytes appeared
highly elongated and extended their processes in the direction of the grooves and along groove
boundaries. Therefore, it is not just the individual cues but a combination of chemical (adsorbed
laminin) and physical (the micropatterned substrate) cues that are required for directional guidance of
astrocytes. Integration of the biological influence of the aligned astrocytes and the physical and
chemical cues presented can potentially generate a permissive environment for specific cellular
behavior such as the orientation and selective differentiation of neural stem cells as well as directed
axon outgrowth during development and regeneration. The understanding gained from this integration
of guidance cues to potentially control neural stem cell outgrowth and differentiation has applications
in guided nerve regeneration within the central nervous system.

3.6 Acknowledgments

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State University for their helpful advice and suggestions.
Table Caption

Table 1.
A summary of the experimental design used in the analysis of astrocyte behavior on 10/20/3 μm patterned and non-patterned polystyrene (PS) substrates.

Figure Captions

Figure 1. The steps involved in the photolithography process following the creation of a photomask and surface preparation of the silicon wafers. These photolithographic techniques allow the preparation of silicon wafers having desired micropatterns. PR = photoresist, M = metal, Si = Silicon Wafer.

Figure 2. A schematic of the growth chamber set up inside a sterile 35 mm petri dish with the polystyrene (PS) substrate, having both patterned and non-patterned sides, adhered to the center of the area bounded by the o-ring. Laminin was applied inside the area bound by the o-ring. Cells were also seeded inside this region.

Figure 3. (A) A 20/10/3 μm silicon wafer used as microdie. (B) An SEM image of a polystyrene film having groove dimensions of 10/20/3 μm created by solvent casting onto the microdie. Scale bars = 20 μm.

Figure 4. (A) Fluorescent image of FITC-conjugated laminin distribution on a 10/20/3 μm micropatterned PS substrate where FITC-conjugated laminin is lining the walls and base of the groove region. (B) Confocal image of the cross section of the FITC-conjugated laminin on micropatterned (10/20/3 μm) PS substrates. A three-dimensional (3-D) representation of the PS substrate was reconstructed showing laminin concentrated at the base of the groove regions. Scale bar = 30 μm.

Figure 5. Expression of astrocytic marker by enriched astrocytes cultures on laminin coated micropatterned (10/20/3 μm) PS substrates (grooves at 90°). (A, B) Fluorescent images of phalloidin (PHALL) stained F-actin and nuclei of astrocytes stained with DAPI and immunoreactivity for (C, D) mGFAP. (D) Merged image created by superimposition of mGFAP and DAPI fluorescence images. Images were taken from PS substrate fixed 24 hours after seeding. Scale bar = 60 μm.

Figure 6. The distribution of the astrocyte orientation on 10/20/3 μm patterned and non-patterned PS substrates (without laminin) for an initial seeding density of 20,000 cells per cm². The data were grouped in 10 degree sectors from 0° to 90°. Data shown are mean values ± 1 standard deviation of N = 372 (individual astrocyte measurements) for NO LAM-NO PATT and N = 255 for NO LAM-PATT.

Figure 7. The effect of surface topography on astrocyte orientation on PS substrates. For an initial seeding density of 20,000 cells per cm², the 10/20/3 μm pattern had a significant effect on the orientation of the astrocytes with respect to the groove direction. The percentage of astrocytes aligning within ≤ 10° and ≤ 20° of the groove direction is demonstrated for 24 and 72 hours. Error bars represent ± 1 standard error of the estimate of the mean (s.e.m.) percentage.
of cells. Standard errors for 5 substrates are reported here (N = 5 for NO LAM-PATT and N = 5 for NO LAM-NO PATT) for each test: ≤ 10° and ≤ 20°). Statistically significant differences are marked with an asterisk (*P < 0.05).

Figure 8. The effect of laminin (0.01 mg/ml in EBSS) on the number of astrocytes adhering to the 10/20/3 μm patterned and non-patterned substrates PS substrates for an initial seeding density of approximately 20,000 cells per cm². The mean cell density was estimated for cell adhesion after 24 hours. A ln transformation was used for statistical analysis of cell count per substrate area and the values were retransformed for presentation here. Data shown are mean values ± s.e.m. (N = 6 for Laminin and N = 6 for No Laminin). Statistically significant differences are marked with an asterisk (*P < 0.05). All laminin coated substrates had significantly higher cell densities than those without laminin.

Figure 9. Astrocytes on 10/20/3 μm patterned PS substrates (grooves at 0°). (A) Fluorescent image of astrocytes seeded on a laminin coated (0.01 mg/ml in EBSS) patterned PS substrate. Astrocytes were spread out on these substrates and aligned in the direction of the grooves. (B) Light microscopy image of the laminin coated patterned PS substrate onto which the cells were seeded. (C) Fluorescent image of astrocytes seeded onto a patterned PS substrate without laminin. A lower proportion of astrocytes aligned on these substrates due to clustering of the cells. (D) Light microscopy image of the patterned PS substrate without laminin onto which the cells were seeded. (A and C) Cells were labeled with CFDA SE in cell suspension prior to seeding. Images were taken from PS substrate fixed 72 hours after seeding. (A-D) Scale bars = 20 μm.

Figure 10. Astrocytes cultured on a laminin coated (0.01 mg/ml in EBSS) PS substrate. On the 10/20/3 μm LAM-PATT/ LAM-NO PATT substrate, astrocytes are aligned in the direction of the groove on the patterned side (grooves at 90°; right of the arrows) while astrocytes were oriented randomly on the non-patterned side (left of the arrows) of the substrate. Astrocytes were stained with CFDA SE in cell suspension prior to seeding. Images were taken from PS substrate fixed 24 hours after seeding. Scale bar = 30 μm.

Figure 11. The distribution of the astrocyte orientation on 10/20/3 μm patterned and non-patterned PS substrates in the presence of laminin (0.01 mg/ml in EBSS) for an initial seeding density of 20,000 cells per cm². The data were grouped in 10 degree sectors from 0° to 90°. Data shown are mean values ± 1 standard deviation of N = 1287 (individual astrocyte measurements) for LAM-NO PATT and N = 1325 for LAM-PATT.

Figure 12. The effect of physical and chemical guidance cues on astrocyte orientation on PS substrates. For an initial seeding density of 20,000 cells per cm², the laminin coated (0.01 mg/ml in EBSS) patterned (10/20/3 μm) substrate had a significant effect on the orientation of the astrocytes with respect to the groove direction. The percentage of astrocytes aligning within ≤ 10° and ≤ 20° of the groove direction is demonstrated for 24 and 72 hours. Error bars represent ± s.e.m. percentage of cells (N = 6 for LAM-PATT and N = 6 for LAM-NO PATT for each test: ≤ 10° and ≤ 20°). Statistically significant differences are marked with an asterisk (*P < 0.05).

Figure 13. Effect of laminin on astrocyte adhesion to 10/20/3 μm LAM-PATT/ LAM-NO PATT PS substrates. The mean cell densities on laminin coated (0.01 mg/ml in EBSS) substrates
versus substrates without laminin were estimated for initial seeding densities of approximately 7500, 13,000, 20,000 cells per cm$^2$. The data presented here is for cell adhesion after 24 hours and was pooled across LAM-PATT and LAM-NO PATT substrates. Data shown are mean values ± s.e.m. ($N = 8$ for 7500 cells per cm$^2$; $N = 10$ for 13,000 cells per cm$^2$; $N = 12$ for 20,000 cells per cm$^2$). Statistically significant differences are marked with an asterisk ($P < 0.05$).

Figure 14. The effects of chemical and physical guidance across initial seeding densities of 7500, 13,000, and 20,000 cells per cm$^2$. The percentage of astrocytes aligning within $\leq 10^\circ$ and $\leq 20^\circ$ of the groove direction (on 10/20/3 μm patterned substrates) is demonstrated for 24 and 72 hours. The data presented here was pooled across all seeding densities and time periods analyzed. Data shown are mean values ± s.e.m. ($N = 24$ for NO LAM-PATT and NO LAM-NO PATT and $N = 36$ for LAM-PATT and LAM-NO PATT for each test: $\leq 10^\circ$ and $\leq 20^\circ$). Statistically significant differences are marked with an asterisk ($P < 0.05$).
Table 1.

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Figure 1.

Insoluble in developer
Soluble in developer

(1) M → Si → (2) PR M → Si → (3) UV lamp

(4) PR M → Si → (5) PR M → Si → (6) mask

(7) → (8) → (9)
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.
Figure 11.
Figure 12.
Figure 13.
Figure 14.
3.7 References


CHAPTER 4

DIRECTED GROWTH AND SELECTIVE DIFFERENTIATION OF NEURAL PROGENITOR CELLS ON MICROPATTERNED POLYMER SUBSTRATES

A paper published in Biomaterials

Jennifer B. Recknor, Donald S. Sakaguchi, Surya K. Mallapragada

4.1 Abstract

Directional growth and differentiation of adult rat hippocampal progenitor cells (AHPCs) were investigated on micropatterned polymer substrates in vitro. Astrocytes or AHPCs cultured on micropatterned polystyrene substrates chemically modified with laminin exhibited over 75% alignment in the groove direction. AHPCs co-cultured with astrocytes preferentially acquired neuronal morphology, with nearly double the percentage of cells expressing class III β-tubulin on the micropatterned half of the substrate, as opposed to the planar half of the substrate, or compared to those growing in the absence of astrocytes. This indicates that substrate three-dimensional topography, in synergy with chemical (laminin) and biological (astrocytes) guidance cues, facilitates neuronal differentiation of the AHPCs. Through multi-dimensional cell-cell interactions, this environment provides spatial control selectively enhancing neuronal differentiation and neurite alignment on topographically different regions of the same substrate. Integrating these cues is important in understanding and controlling neural stem cell differentiation and designing scaffolds

1 Reprinted with permission of Biomaterials, Copyright © 2006, in press.
2 Primary researcher and author, Department of Chemical Engineering and the Neuroscience Program, Iowa State University
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for guided nerve regeneration.

4.2 Introduction

Axonal regeneration in the central nervous system (CNS) is limited by inhibitory influences of the glial and extracellular environment [1-3]. Experimental strategies employing neural stem cell (NSC) transplantation hold great promise for repairing the injured and diseased CNS. NSCs are self-renewing and multi-potent producing differentiated, functional progeny including neurons and glial phenotypes [4, 5]. NSCs, as well as neural progenitor cells (NPCs), which have more limited capacities in terms of growth and differentiation, have been isolated and expanded from the developing and adult CNS in a variety of mammalian species, including humans [5-7].

Efforts have been made in vitro to elucidate the stem cell microenvironment, or ‘niche’, controlling cell fate in vivo [4, 8, 9]. Studies have demonstrated that the fate of differentiating stem cells is strongly influenced by direct cell-to-cell and cell-to-extracellular matrix (ECM) contacts involving a complex “cocktail” of growth factors, signaling molecules, and ECM proteins [10-12]. Furthermore, astrocytes have been studied as influential components of an ‘instructive’ stem cell niche [13] and can induce neurogenesis in NSCs [8, 13-16].

Another promising strategy providing instructive environments for axonal regeneration and restoration of function involves scaffolds or bridging substrates [17, 18]. Such substrates can be designed with specific microarchitectures that allow optimal structural support for axonal regrowth and affect cellular orientations and the presentation of ECM proteins to the cells. These guidance channels have been used to study NSC behavior for application in the treatment of spinal cord injury and neurodegenerative diseases of the CNS [17-27]. Together, these studies support the notion that synthetic materials can provide mechanical scaffolds supporting NSC growth and differentiation. However, to our knowledge, the synergistic effects of multiple stimuli involving physical or topographical cues, in conjunction with chemical and biological cues on NPC differentiation have not
been explored, and the mechanisms by which this combination of cues affects cellular differentiation are unknown.

Combining physical, chemical and biological guidance cues that enable spatial control over NPC differentiation on topographically different regions of the same substrate can potentially generate a supportive environment for eliciting regeneration and restoring function in the injured or diseased CNS. Integrating multiple stimuli to direct the lineage of endogenous or engrafted CNS-derived precursor cells on specific substrate regions offers opportunities to mimic the natural in vivo environment and elucidate the mechanisms behind efficient stem cell-mediated repair of the CNS that cellular transplantation or scaffolds alone do not provide. In the present study, the effects of these cues on directing alignment and spatially controlling the differentiation of adult rat hippocampal progenitor cells (AHPCs) were investigated. Postnatal rat type-1 astrocytes [28] and AHPCs extended axially along the grooves of micropatterned polystyrene (PS) substrates chemically modified with laminin. The biological influence of astrocytes was integrated with the physical (micropatterned substrate) and chemical (laminin) guidance cues, and the synergistic effects of these cues on AHPC differentiation was compared to cells not exposed to physical and/or biological guidance cues. This research provides insights into mechanisms of NSC differentiation and a foundation for a promising regeneration strategy for guided CNS repair.

4.3 Materials and Methods

4.3.1 Micropatterned substrate fabrication

Polystyrene (PS) was chosen for substrate fabrication, as it is a biocompatible polymer that is used extensively in cell culture experimentation. Conventional photolithographic techniques and reactive ion etching were used to fabricate silicon wafers with the desired micropatterns that were then transferred to the polymer substrates using solvent casting [28]. The patterns used were described by: groove width (μm)/ groove spacing (or mesa width) (μm)/ groove depth (μm). To study
the physical guidance of AHPCs on micropatterned PS substrates, the pattern dimensions used for these experiments were 16/13/4 µm.

The solvent cast polymer substrates were fabricated from an 8% (w/v) PS (MW 125,000-250,000) (Polysciences, Inc., Warrington, PA) solution in toluene. Substrate thicknesses of approximately 50-70 µm were achieved using solvent casting techniques. After casting and drying for a minimum of 24 hours, the PS substrate was removed by soaking in deionized (DI) water and then sterilized with 70% ethanol. The micropatterned substrates were imaged using scanning electron microscopy (SEM). After mounting, the samples were sputter coated (SEM Coating Unit E5100, Polaron Instruments, Inc., Watford Hertfordshire, UK) with gold and imaged using a JEOL JSM-840A at an accelerating voltage of 20 kV, a 50 µm diameter aperture and a vacuum level of 1 x 10⁻⁶ Torr.

Cell growth chambers were constructed using PTFE (Teflon®) o-rings (Small Parts, Inc., Miami Lakes, FL), glass coverslips, and PS substrates (1 cm² in area) as described previously[28]. The PS substrates were coated with poly-L-lysine (PLL; Sigma, St. Louis, MO) solution at a concentration of 100 µg/ml and/or laminin (LAM, Sigma) at a concentration of 10 µg/ml in Earle's Balanced Salt Solution (EBSS; Gibco, Grand Island, NY).

4.3.2 Astroglial cell isolation and purification

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. A population of purified cortical astrocytes was obtained from neonatal rat pups as described in Recknor et al.[28] Briefly, cerebral hemispheres were freshly dissected from 1-3 day old Sprague-Dawley rat pups and treated with papain solution (20 IU/ml; 37 °C, 5% CO₂/95% air, 1 hour) (Sigma). After subsequent treatment with trypsin inhibitor solution (10 mg/ml; Sigma), the tissue was mechanically dissociated in modified minimal essential culture medium (MMEM). The cultures were grown to confluence in 25 cm² tissue culture flasks (T-
25; Falcon) at 37 °C in a humidified 5% CO₂/95% air atmosphere. The culture medium, MMEM, consisted of minimum essential medium (MEM; Gibco) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate and 14 mM NaHCO₃, penicillin (100 IU/ml) and streptomycin (100 µg/ml) with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT), pH 7.35.

Enriched type-1 astrocyte cultures were prepared as previously described [28]. After the cultures reached confluency (~8 days), the cells were shaken twice on a horizontal shaker at 260 RPM at 37 °C, first for 1.5 hours and then for 18 hours. The remaining adherent cells were enzymatically detached with trypsin (0.1% in EBSS; Sigma), pelleted (100 X g, 10 min.), resuspended in MMEM and passaged into 25 cm² tissue culture flasks. Cultures were fed every 3 days and were not passaged more than 8 times. Over 90% of the cells cultured under these conditions were selectively labeled by GFAP antibody (data not shown) confirming their astrocyte identity.

4.3.3. Adult hippocampal progenitor cell culture

Adult rat hippocampal progenitor cells (AHPCs) were originally isolated from the brains of adult Fischer 344 rats as reported by Palmer and colleagues [6]. The expanded cultures of single clones were infected with retrovirus to express enhanced GFP [6, 29]. The AHPCs were maintained in plastic tissue culture flasks (T-75, Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 µg/ml; Sigma) and mouse-derived laminin (5 µg/ml; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4). The AHPCs were maintained in complete medium containing Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and L-glutamine (2.5 mM L-glu; Gibco BRL, Gaithersburg, MD). For in vitro analysis on PS substrates, the AHPCs were detached from the T-75 flask using ATV solution (Gibco BRL, Gaithersburg, MD), harvested and collected by centrifugation.
at 1000g for 5 minutes. The pellets were resuspended in the culture medium stated above without FGF (referred to as differentiation medium) or co-culture medium (described below) and triturated gently. The cells were then plated on the micropatterned PS substrates coated with PLL (100 μg/ml in borate buffer) and laminin (10 μg/ml EBSS) (PS-LAM substrates) at initial densities of 10,000 to 15,000 cells/cm². Cells were maintained at 37 °C in a 5% CO₂/95% air atmosphere for 6 days in culture medium.

4.3.4 Co-culture of astrocytes and AHPCs

Purified astrocytes were seeded onto PS-LAM substrates inside growth chambers and cultured for 2 days to generate near confluent monolayers. AHPCs were plated on top of the astrocyte monolayer at approximately 15,000 cells/cm². The co-cultures were maintained in a mixed medium that consisted of astrocyte MMEM (without FBS) in a 1:1 mixture with AHPC differentiation media (referred to as co-culture media). As controls, AHPCs and astrocytes were plated in the same co-culture medium at the same density. AHPC-astrocyte co-cultures, AHPCs and astrocytes were grown for 6 days and then fixed with 4% paraformaldehyde in 0.1 M PO₄ buffer (pH = 7.4).

4.3.5 Analysis of AHPCs In Vitro: Immunocytochemistry

Cells cultured on micropatterned/non-patterned PS-LAM substrate surfaces were processed for immunocytochemistry according to standard protocols described previously[28]. Briefly, cells were first rinsed in 0.1 M PO₄ buffer, fixed using 4% paraformaldehyde in 0.1 M PO₄ buffer, rinsed, and processed. Cultured cells were incubated in blocking solution (3-5% donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% TritonX-100 (Fisher Scientific)) for 45 minutes. Specific primary antibodies (see Antibodies section) were used to identify differentiated neurons and glia. Cells were incubated in primary antibodies overnight at 4°C in a humid chamber, washed in potassium PBS (KPBS, 0.15 M NaCl, 0.034 K₂HPO₄, 0.017 KH₂PO₄, pH 7.4) with Triton-X,
incubated in the appropriate biotinylated secondary antibodies for 2 hours, rinsed, and incubated with streptavidin Cy3 (Jackson ImmunoResearch, West Grove, PA) in the dark for 30 minutes. The cells were then rinsed and stained with 4’, 6-diamidino-2-phenylindole, dilactate (DAPI), a semi-permeant nucleic acid stain. DAPI was diluted at 1:100 in PBS and applied for 15 minutes. Preparations were rinsed and then mounted onto microscope slides using an antifade mounting medium (Gel Mount; Biomedica Corp., Foster City, CA).

Cells were observed using light microscopy (Olympus IMT-2 bright field/phase contrast microscope) and epifluorescence microscopy (Nikon Corp., Melville, NY) during culture. Digital images were taken throughout experimentation using a Nikon Eclipse (Nikon Corp.) inverted microscope equipped with standard epifluorescence illumination and differential interference contrast (DIC) optics equipped with a cooled digital camera (ORCA, Hamamatsu) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA). Cultured AHPCs were also examined using a photomicroscope (Microphot FXA; Nikon Corp.). Images were captured with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a frame grabber (Megagrabber; Perceptics, Knoxville, TN, in a Macintosh computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Some preparations were visualized and images captured using a confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc., Exton, PA). Some preparations were visualized using SEM. After mounting, the samples were sputter coated with gold-palladium using a Denton Desk II Sputter Coater (Denton Vacuum, Inc., Moorestown, NJ). Images were collected using a JEOL JSM-5800LV scanning electron microscope at 10 kV (Japan Electron Optic Laboratory, Peabody, MA) with the ADDA II digital image capture system and SIS Pro software (Soft Imaging System Inc., Lakewood, CO).
4.3.6 Quantitative analysis of immunocytochemistry

Following immunocytochemical procedures on PS-LAM substrates, the preparations were examined and photographed using fluorescence microscopy (Nikon epifluorescence microscope with Hamamatsu digital camera). A 20X objective was used to examine twelve microscope fields, each field representing 0.24 mm² (546 μm by 438 μm). Six fields were examined from the micropatterned half and non-patterned half of the PS-LAM substrates. The following counts were made in each field: the total number of cells (DAPI stained nuclei), the number of GFP⁺ cells and the number of cells expressing the primary antibody of interest, TuJ1, RIP, or GFAP (see Antibodies section). These data were used to calculate the percentage of cells labeled with DAPI and with one of the antibody markers (mentioned above) on each PS-LAM substrate. The data collected after 6 days for each antibody was compared and analyzed. The experiment was repeated three to four times with two substrates per treatment in each experiment.

4.3.7 Antibodies

Differentiated neurons were identified using an antibody directed against class III β-tubulin (TuJ1 mouse IgG, Research Diagnostics, Inc.) diluted 1:750. Anti-glial fibrillary acidic protein (GFAP; Anti-GFAP, mouse IgG; ICN) diluted at 1:600 and polyclonal anti-GFAP antibody (rabbit IgG, Sigma) diluted at 1:100 were used as markers of astrocytes. Terminally differentiated oligodendrocytes were identified using Anti-RIP (1:1600) (mouse IgG, obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biology, University of Iowa, under contract NO1-HD-2-3144 from the NICHD). Biotinylated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) was used at a dilution of 1:500. Donkey anti-rabbit secondary antibody conjugated with Cy 5 (Jackson ImmunoResearch) was used at a dilution of 1:150. All primary and secondary antibodies were diluted in blocking solution. Streptavidin Cy3 was diluted in
PBS to 1:15,000. Negative controls were performed in parallel by omission of the primary and secondary antibodies. No antibody labeling was observed in the controls.

4.3.8 Determination of cell alignment

Cultured cells on the PS substrates were fixed, labeled and mounted onto glass microscope slides (Fisher Scientific, Pittsburgh, PA). The cells were examined and photographed using fluorescence microscopy (Nikon epifluorescence microscope with Hamamatsu digital camera) with a 20X objective, each field representing 0.24 mm² (546 µm by 438 µm). The orientation of the AHPCs on the PS-LAM substrates was measured quantitatively using MetaMorph software (Universal Imaging Corp., West Chester, PA) as the angle of the longest chord through each AHPC (and the processes surrounding the cell) relative to the horizontal axis of the imported image. The data were grouped in 10° sectors between -90° to 90°. Orientations of the groove position in the images were measured in the same way. Control data were taken from measurements made on AHPCs on non-patterned PS substrate areas adjacent to the patterned areas. The angle of orientation in these controls was measured relative to the horizontal axis (0°). Statistical analysis was performed on the values of the differences between the orientation of the cell and the orientation of the groove on the substrate with a difference of 0° indicating perfect alignment. Cell alignment was measured as the proportion of AHPCs whose longest chord makes an angle of ≤ 20° with the direction of the grooves in these studies. The proportions of cells falling in this group were estimated for both micropatterned and non-patterned PS-LAM substrates. All of the measurements for this analysis were performed on two substrates with 3 regions (or fields) measured on the micropatterned halves of the substrates and 1 region measured on the non-patterned halves of the substrates.
4.3.9 Statistical analyses

Statistical analyses were performed on (1) the AHPC alignment to within 20° of groove direction and (2) on the percentage of cells expressing the antibody of interest. Cell alignment was compared on a success and failure basis. Cell alignment was considered a ‘success’ when the longest cord of the cell was within 20° of the direction of the grooves on the substrate and a ‘failure’ otherwise. All of the measurements for this analysis were performed on two substrates. The mean percentage of successes was calculated and mean differences were taken between the patterned and non-patterned halves of both substrates. Analysis was then performed using a paired t-test. The percentage of cells expressing the antibody of interest was also analyzed. Six regions (or fields) were examined from the micropatterned halves and non-patterned halves of the PS-LAM substrates. Since each region was a subsample of that half of the substrate, the means of the regions were calculated for use in the analysis so that each region would receive equal weight. The experiment followed a split plot design since the treatment (co-culture or control) was applied to the entire substrate and both the micropatterned and non-patterned regions were within one substrate. Due to this design, there were two random effects in the model, (1) for the whole plot effect, consisting of the error term for the treatment and (2) for the split plot effect, which included the pattern effect and the interaction between the treatment and pattern. The whole plot experimental unit was the entire PS-LAM substrate (approximately 1 cm² in area) while the split plot experimental unit was the half of the substrate that was either patterned or non-patterned. For TuJ1, n=9 for the whole plot analysis and n=18 for the split plot analysis. For RIP, n=7 for the whole plot analysis and n=14 for the split plot analysis. Mixed model analysis was performed on the means using the PROC MIXED procedure in SAS. All tests performed were two-sided tests and p-values less than an alpha value of 0.05 were considered significant. Analysis of the residuals was performed and there was no evidence of assumption violations.
4.4 Results

A technique for fabricating microgrooves on PS substrates was developed, and the effects of these three dimensional (3-D) patterns on AHPC behavior in vitro were investigated. Cells were cultured on square substrates having 0.5 cm$^2$ of patterned substrate (Figure 1) adjacent to 0.5 cm$^2$ of non-patterned substrate (as a control). To study the physical guidance of AHPCs on micropatterned PS substrates, the pattern dimensions optimized for these experiments were 16/13/4 μm (groove width/ groove spacing (or mesa width)/groove depth), which were based on previous studies involving the alignment of astrocytes, Schwann cells and neurites [28, 30-32]. In synergy with the physical guidance of the micropatterned PS substrate, chemical and biological cues were incorporated into this culture system to investigate the influence of multiple cues on directing AHPC growth and differentiation.

4.4.1 Effect of guidance cues on the alignment of AHPCs

AHPCs were cultured on PS substrates with micropatterned/non-patterned surfaces coated with laminin (PS-LAM) to determine the influence of substrate-mediated contact guidance on growth and differentiation. It has been previously demonstrated in vitro that a combination of physical and chemical guidance cues resulted in significant alignment of astrocytes on the PS-LAM substrates [28]. The morphology and alignment of the AHPCs in co-culture with astrocytes on the micropatterned substrates was compared to those growing on planar surfaces or in the absence of astrocytes.

Under differentiation conditions, the AHPCs adopted various morphologies on PS-LAM substrates. Cells appeared unipolar, bipolar or multipolar having characteristics of neurons or glial cells. AHPCs exhibiting neuronal morphologies had highly elongated processes oriented parallel to the grooves of the patterned substrate (Figure 2a). The cells on the non-patterned surfaces did not exhibit a particular bias in alignment, extending processes in a radial fashion (Figure 2b). AHPC
growth and orientation on the micropatterned surface was influenced by the 3-D topography of the substrate regardless of the phenotype expressed in differentiation conditions. The patterned substrate directed the AHPCs to extend processes along the inside of the groove, on the mesas, or at the boundary between the groove and the mesa and influenced cells to spread in the groove direction. Cell alignment was determined by whether the longest chord through each individual AHPC made an angle of ≤ 20° with the direction of the grooves. The distributions of cell orientations (taken as the difference between the AHPC orientation and the groove orientation) on the micropatterned and non-patterned substrates are displayed in Figure 3. The results indicate that over 75% of the AHPCs aligned within 20° of the groove direction on the micropatterned substrates while the AHPCs growing on the non-patterned substrates were randomly oriented (n = 2; α = 0.05 and p = 0.01).

To investigate the synergistic influence of physical, chemical and biological cues on NPC differentiation, AHPCs were co-cultured with cortical astrocytes on the PS-LAM substrates. In co-cultures, AHPC processes appeared oriented in the groove direction on the micropatterned PS-LAM substrates with many AHPCs exhibiting characteristic neuronal morphologies (Figure 4a). On non-patterned substrates, AHPC outgrowth was random as were the astrocyte orientations (Figure 4b). On the patterned substrates, AHPC outgrowth appeared to be influenced by the presence of aligned astrocytes with processes extending along astrocyte processes, though the significant alignment seen in the case of AHPC cultures alone was not seen in the co-cultures. Many AHPCs in contact with astrocytes elaborated processes that extended parallel to the grooves (Figures 4c-d). As the majority of the AHPCs were not in direct contact with the micropatterned PS-LAM substrates, it appears that the observed oriented growth was influenced by cues at or near the surface of the (aligned) astrocytes.

4.4.2 Effect of guidance cues on the differentiation of AHPCs

AHPCs were morphologically and immunocytochemically characterized on the micropatterned/non-patterned PS-LAM substrates at 6 days in vitro using cell type specific
antibodies. The phenotypes of AHPCs growing under differentiation conditions in co-culture media were assessed using antibodies directed against class III β-tubulin (TuJ1), a protein characteristic of early neurons, and the glial markers, receptor interacting protein (RIP) and glial fibrillary acidic protein (GFAP), for oligodendrocytes and astrocytes, respectively. Counterstaining with DAPI allowed visualization of the nuclei. The percentages of AHPCs immunoreactive (IR) for TuJ1 and RIP in co-culture and in the absence of astrocytes on the patterned and non-patterned sides of the PS-LAM substrates are displayed in Figure 5. Approximately 16% of AHPCs cultured in the absence of astrocytes were TuJ1-IR cells displaying neuronal morphologies and exhibiting neurites of various lengths (Figure 6a-f). On the micropatterned side, TuJ1-IR processes were highly aligned in the groove direction (Figure 6a-c). It was determined, however, that the percentage of AHPCs immunoreactive for TuJ1 on the patterned substrates was not significantly different than that on the planar substrates (α = 0.05; p = 0.52). Approximately 20% of the AHPCs expressed RIP and exhibited oligodendrocyte-like morphologies with many slender processes radiating out from their cell bodies (Figure 6g-l). On the micropatterned substrates, process outgrowth was more directed with RIP-IR cells having membranous extensions that aligned inside the grooves (Figure 6g-i). There was no significant difference in the number of RIP-IR cells on the patterned as compared to the non-patterned surfaces (α = 0.05; p = 0.72).

After six days in vitro, a significantly greater proportion of the AHPCs in co-culture were TuJ1-IR on the micropatterned substrate (35.3%) compared to the planar substrate (21.8%) (α = 0.05; p = 0.0003). In addition, the results showed that, on the planar surfaces, while approximately 20% of the AHPCs were TuJ1-IR in the co-cultures and approximately 16% were TuJ1-IR in the absence of astrocytes, there was no evidence of a significant difference between the two conditions (α = 0.05; p = 0.13). However, on the micropatterned surfaces, the percentage of TuJ1-IR AHPCs in the co-cultures (35.3%) was significantly higher than that in the absence of astrocytes (15.3%) (α = 0.05; p = 0.0004). Furthermore, AHPCs in co-culture on the micropatterned surfaces appeared more mature...
extending long, elaborate TuJ1-IR processes in the direction of the grooves and astrocyte cytoskeletal filaments (Figure 7a-f). Thus, it was apparent that both AHPC morphology and phenotype were influenced by the 3-D topography of the patterned substrate as well as the presence of the aligned astrocytes. Approximately 25% of the AHPCs in co-culture expressed RIP on either the patterned or non-patterned surfaces. On the micropatterned substrates, RIP-IR cells elaborated extensive processes weaving intricately inside the grooves and along the mesas of the substrate (Figure 7g-i). On the non-patterned substrates, radial outgrowth from RIP-IR cells was observed (Figure 7j-l). There was no evidence of a significant difference in the percentage of RIP-IR cells on the patterned and planar substrates or in co-culture compared to AHPCs cultured in the absence of astrocytes (α = 0.05; p = 0.27 and p = 0.052, respectively). GFAP-IR cells displaying flattened morphologies with large nuclei were rare in all conditions examined and composed less than 0.1% of immunoreactive cells.

4.5 Discussion

Our results demonstrate that micropatterned PS-LAM substrates cultured with astrocytes serve as effective guidance substrates for selectively enhancing neuronal differentiation and directing the growth of AHPCs. On these substrates, astrocytes were observed as having flattened morphologies with F-actin microfilaments and glial intermediate filaments elongated in the groove direction [28]. AHPCs also extended highly aligned processes on the patterned surfaces. Furthermore, the combination of physical, chemical and biological guidance cues facilitated and instructed differentiation of the AHPCs into cells acquiring neuronal morphology that were immunoreactive for TuJ1, when compared to cells not exposed to physical and/or biological guidance cues. Through multi-dimensional cell-cell interactions, this integrated co-culture environment provided dynamic spatial control over AHPC behavior as demonstrated by enhanced neuronal differentiation and oriented neurite outgrowth on the micropatterned regions of the substrate.
4.5.1 Effect of scaffolds on NSC

Providing a 3-D support for cell growth enables control of cell function, interaction and spatial and temporal guidance of the complex processes of tissue formation and regeneration [33]. In previous studies, scaffolds have been employed to direct the organization of NSCs and NPCs in vitro or in vivo and to attract endogenous progenitor cells and induce their differentiation towards a desired pathway [19-26]. By applying multiple stimuli to the AHPC environment, we have incorporated more than just mechanical support for cell growth. Through the influence of aligned astrocytes, we have achieved biological and spatial control over AHPC behavior creating a dynamic 3-D microenvironment that has significantly enhanced neuronal differentiation of AHPCs. Neuronal differentiation among AHPCs increased nearly two-fold in co-cultures on the micropatterned surface versus the non-patterned surfaces and AHPCs in the absence of astrocytes. Furthermore, neurites were oriented in the groove direction and along aligned astrocytes.

4.5.2 Effect of astrocytes on NSCs

It has been shown that local environments have a profound influence on the fate of NSCs. Numerous in vitro and in vivo studies have demonstrated that astrocytes can stimulate the proliferation of NPCs [8, 16, 34]. Recently, Song and colleagues observed astrocytes actively regulating neurogenesis from AHPCs and that neonatal and adult hippocampal astrocytes retain the ability to promote neurogenesis [8]. Their results demonstrated that regionally specified astroglial cells provide a unique niche for adult neurogenesis and have the potential to function as cues for the differentiation of FGF-2 dependent AHPCs [8]. Furthermore, astrocytes have had similar effects on NSCs derived from different brain regions. Kornyei and colleagues have shown that astrocytes induce neuronal differentiation of neuroectodermal progenitors through direct cell-to-cell contacts and short-range acting humoral factors [14]. They also established that the signals inducing neuronal differentiation were conserved among various mammalian species [14]. Our results provide insight
into the mechanisms behind NPC differentiation suggesting not only an active role for astrocytes in adult brains but also the instructive role of spatial control provided by the integration of micropatterned substrates and aligned astrocytes on these substrates. On the PS-LAM substrate, cortical astrocytes supported neuronal differentiation of AHPCs regardless of the substrate surface. However, the micropatterned 3-D environment incorporating the astrocytes enhanced neuronal differentiation of the AHPCs significantly. Furthermore, while there was not a significant difference in TuJ1 immunoreactivity among AHPCs in co-culture versus AHPCs in the absence of astrocytes on the planar surfaces, a significantly higher percentage of TuJ1-IR AHPCs was observed on the patterned surfaces in the presence of astrocytes. This demonstrates strong evidence that the 3-D patterned surface influences astrocyte morphology and provides guidance cues for inducing neuronal differentiation that astrocytes alone cannot provide. Based on previous work by Song and colleagues [35] revealing a basal level of neurogenesis among adult NPCs in the presence of astrocytes, it is possible that the patterned substrate may increase progenitor cell proliferation leading to this enhanced neuronal differentiation. However, cell counts have revealed approximately the same number of AHPCs in co-cultures on the micropatterned and non-patterned PS-LAM substrates suggesting that differential neurogenesis between the two surfaces is unlikely. This is further evidence that the synergistic combination of the spatial control of the micropatterned substrate and the biological influence of the astrocytes leads to neuronal differentiation of AHPCs.

### 4.5.3 Interactions of NSCs with their environment

Integrins, ECM molecules, and cell adhesion molecules, have important roles in CNS development inducing, coordinating, and regulating many complex cellular processes including cell growth, differentiation, migration, survival, proliferation, tissue organization and matrix remodeling [36-39]. It has been observed in vitro and in vivo that various ECM components regulate the differentiation of NSCs [9, 12, 40]. Our results demonstrate that the interaction between the
micropatterned surface and the presence of the longitudinally aligned astrocytes effectively promotes oriented growth and induces significant neuronal differentiation of adult NPCs. We propose that, in this multi-dimensional environment, the aligned astrocytes present discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both. These factors may include specific molecules, originating from the extracellular microenvironment, known to mediate cellular proliferation, differentiation, process outgrowth and adhesion of neurons to astrocytes [41-43]. Astrocytes have been shown to produce a variety of soluble and membrane-associated factors, including cytokines and neurotrophic factors, which stimulate the viability and proliferation of many distinct cell types and influence multiple CNS functions [44]. It has further been demonstrated that aligned astrocytes can secrete linear arrays of ECM and adhesive proteins and influence the direction and length of outgrowth of neurites [45].

While the exact mechanism cannot be deduced from the present study, our current data indicate that the distribution of active neurite outgrowth-promoting molecules presented by the aligned astrocytes on the micropatterned PS-LAM substrates created a multi-dimensional microenvironment that instructed neuronal differentiation among the adherent NPCs. Multiple interactions with the microenvironment, or ‘niche’, regulate NPC fate [9]. It is likely that the stem cell niche has been mimicked *in vitro* through the presentation of an optimal combination of signals necessary for neuronal differentiation to the AHPCs by the micropatterned PS-LAM environment. Similar to the extracellular transport processes that are critical to morphogenesis [46], the biological and spatial control over AHPC differentiation achieved by this unique, synergistic combination of cues may be the result of extracellular concentration gradients of molecules presented by aligned astrocytes as well as neighboring progenitor cells in confined channels. The significant difference seen among AHPCs in co-culture and in the absence of astrocytes on the patterned surfaces and not on the planar surfaces suggests that the 3-D environment provides a means for concentrating factors necessary for inducing neuronal differentiation. Microgradients of such molecules may have been
established as the astrocytes aligned and released soluble factors that bind to components of the ECM deposited in the grooves and on the mesas of the micropatterned surface. As the AHPCs interacted with the astrocytes in this 3-D microenvironment, enhanced neuronal differentiation among the AHPCs may have resulted from these localized microgradients.

4.6 Conclusions

The results of this study demonstrate a strategy for enhancing adult NPC differentiation that has important applications in guided nerve regeneration in the CNS. Micropatterned PS substrates chemically modified with laminin were used to study the growth and differentiation of AHPCs. The PS-LAM substrates directed AHPC alignment with cell processes elongated in the groove direction on the patterned surface. In contact co-culture with astrocytes, the synergy among the three-dimensional topography and the chemical (laminin) and biological (astrocytes) guidance cues instructed AHPC differentiation with a significantly higher percentage of cells expressing TuJ1 on the micropatterned half of the substrate, as opposed to the planar half of the substrate, or compared to those growing in the absence of astrocytes. This multi-dimensional microenvironment provided spatial control over differentiation facilitating neuronal differentiation and promoting neurite alignment on topographically distinct regions of the same substrate. It is conceivable that soluble and/or contact mediated cues presented by the aligned astrocytes influenced differentiation as well as the oriented growth of the AHPCs on the astrocyte monolayer. To achieve the desired morphological and functional characteristics essential for neural regeneration, an integrative approach, employing NPCs with the proper biological, biochemical and biophysical signaling, such as the multiple stimuli presented in this paper, is necessary. The dynamic microenvironment that results from this synergistic combination of guidance cues could be applied as a tissue engineering strategy in which cells and signaling molecules are combined first in vitro, to create a biomaterial suitable for transplantation. Creating an environment that incorporates multiple guidance cues to direct and selectively instruct the
terminal lineage of endogenous or engrafted NSCs offers exciting opportunities for elucidating the mechanisms behind cellular differentiation, nerve regeneration and specific considerations for the efficient stem cell-mediated repair of the CNS.

4.7 Acknowledgments

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Figure 1. A scanning electron microscopy image of a micropatterned PS substrate with groove dimensions 16/13/4 μm (groove width/groove spacing/groove depth). Patterned substrates with these dimensions were used throughout this study. Scale bar = 10 μm.

Figure 2. Scanning electron microscopy (SEM) images of AHPCs cultured on a PLL (100 μg/ml) and laminin (10 μg/ml) coated PS substrate. (a) On the micropatterned substrate, AHPCs elaborated processes that were aligned in the direction of the grooves (oriented at 0°) (b) On the non-patterned (smooth) side of the substrate, AHPC processes were oriented randomly. Images were taken from cultures at 7 days after plating. Scale bar = 30 μm.

Figure 3. Over 75% of the AHPCs aligned within 20° of the direction of the grooves on the micropatterned (Pattern) PS-LAM substrates at an initial plating density of 10,000 cells per cm². No particular bias in alignment was observed on the non-patterned (No Pattern) substrate. The data seen above are grouped in 10° sectors from 0° to 90°. Data shown are mean values (%) ± 1 standard deviation with N = 408 (individual AHPC measurements from 3 regions) for Pattern and N = 157 (individual AHPC measurements from 1 region) for No Pattern.

Figure 4. SEM images of astrocytes and AHPCs co-cultured on PS-LAM substrates. (a) On the micropatterned half of the substrate, AHPC processes were oriented in the groove direction (orientation at 0°) and were randomly oriented on the non-patterned half of the substrate. (b) On the non-patterned side of the substrate, AHPCs on astrocyte (Astro) monolayers exhibited a variety of cellular morphologies with no particular bias in alignment. Arrows point to examples of radially oriented AHPCs. (c) AHPCs were in contact with and extending along astrocyte cytoskeletal filaments with processes aligning in the groove direction. AHPCs were also in contact with the grooves. (d) Inset seen in (c) displaying the elaborate processes of AHPCs (arrows) oriented along a groove on top of the astrocyte (Astro) monolayer. Images were taken from co-cultures at 6 days after plating. (a,b): Scale bars = 50 μm; (c,d): Scale bars = 30 μm.

Figure 5. Differentiation of AHPCs in a serum-free defined co-culture medium (AHPCs only) or in co-culture with neonatal rat astrocytes (Astrocyte-AHPC Co-culture). Cells in six day cultures were stained for markers of neurons (TuJ1) and glial cells (RIP and GFAP). (a) Quantification of TuJ1-immunoreactive (IR) cells. (b) Quantification of RIP-IR cells. GFAP-IR cells were rare in all conditions examined and composed less than 0.1% of IR cells. Data are the percentage of GFP-expressing IR cells, mean ± standard error and represent pooled data from six different regions on each of the patterned and non-patterned halves of separate polystyrene substrates from three to four experiments in parallel cultures. *Significantly different than AHPCs co-cultured on non-patterned (No Pattern) substrates (α = 0.05; p = 0.0003). †Significantly different than AHPCs alone cultured on patterned (Pattern) substrates (α = 0.05; p = 0.00004).

Figure 6. Expression of class III β-tubulin (TuJ1), an early neuronal protein, and RIP, a characteristic oligodendrocyte marker, by AHPCs cultured on micropatterned (Patt) (a-c; g-i) and non-patterned (No Patt) (d-f; j-l) PS-LAM substrates. (a,d) Fluorescent images illustrating GFP-expressing AHPCs and immunoreactivity for (b,e) TuJ1. (c,f) Merged image created by the superimposition of GFP (green), TuJ1 (red) and DAPI nuclear counterstain (blue) fluorescent images. (g,j) Fluorescent images illustrating GFP-expressing AHPCs and immunoreactivity for (h,k) RIP. (i,l) Merged image with DAPI nuclear counterstain (blue).
Images were taken from AHPCs in co-culture media for 6 days. Dotted lines indicate the location of a groove on the micropatterned surface. Scale bars = 15 μm.

Figure 7. Confocal images showing TuJ1 and RIP immunoreactivity in AHPCs co-cultured with astrocytes on micropatterned (Patt) (a-c; g-i) and non-patterned (No Patt) (d-f; j-l) PS-LAM substrates. (a,d) Fluorescent images illustrating GFP-expressing AHPCs and immunoreactivity for (b,e) TuJ1. (c,f) Merged image created by the superimposition of TuJ1 (red), GFAP (magenta) GFP (green) and DAPI nuclear counterstain (blue) fluorescent images. (g,j) Fluorescent images illustrating GFP-expressing AHPCs and immunoreactivity for (h,k) RIP. (i,l) Merged image with DAPI nuclear counterstain (blue). Images were taken from co-cultures at 6 days after plating. Dotted lines indicate the location of a groove on the micropatterned surface. Arrows indicate GFP-expressing AHPCs expressing marker of interest. Scale bars = 30 μm.
Figure 1.
Figure 2.
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Figure 4.
Figure 6.
Figure 7.
4.7 References


CHAPTER 5

MICROENVIRONMENTAL REGULATION OF NEURAL PROGENITOR CELL OUTGROWTH AND DIFFERENTIATION

A manuscript in preparation

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5.1 Abstract

Our previous studies of directional growth and differentiation of adult rat hippocampal progenitor cells (AHPCs) on micropatterned polymer substrates seeded with astrocytes indicated that substrate three-dimensional topography, in synergy with chemical (laminin) and biological (astrocytes) guidance cues, facilitates selective neuronal differentiation of the AHPCs \cite{1}. Through multi-dimensional cell-cell interactions, this environment provides spatial control selectively enhancing neuronal differentiation and neurite alignment on topographically different regions of the same substrate. While the exact mechanism was not deduced from this contact co-culture study, we proposed that the aligned astrocytes presented discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both. In this study, using a non-contact co-culture system, the potential role of astrocyte-derived soluble cues involved in selective differentiation and the mechanism behind the interaction of guidance cues was investigated. Using a Transwell\textsuperscript{6} semi-porous membrane insert to separate the astrocytes from AHPCs cultured on micropatterned substrates in the same well, it was determined that soluble factors can induce

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significant selective neuronal differentiation and neuritic extension among the adherent AHPCs as compared to contact co-cultures. The interaction of these soluble cues with the topographical cues provides an optimal combination of signals for enhanced neuronal outgrowth and differentiation. This synergistic combination of guidance cues is essential for understanding mechanisms of neural stem cell (NSC) differentiation and can be applied to in vivo strategies for facilitating regeneration in the central nervous system.

5.2 Introduction

It has been shown that local microenvironments, composed of multiple cell types and extracellular matrix (ECM) - derived stimuli, have a profound influence on the fate of neural/stem progenitor cells. Numerous in vitro and in vivo studies have demonstrated that astrocytes can stimulate the proliferation of neural progenitor cells (NPCs) [2-5]. Results from our previous studies provide insight into the mechanisms behind NPC differentiation suggesting not only an active role for astrocytes among adult NPCs but also the instructive role of spatial control provided by the integration of micropatterned substrates and aligned astrocytes on these substrates. On the polystyrene micropatterned/non-patterned substrates chemically modified with laminin (PS-LAM), cortical astrocytes supported neuronal differentiation of AHPCs regardless of whether the substrate surface was patterned or non-patterned. However, the micropatterned three dimensional (3-D) environment incorporating the astrocytes enhanced neuronal differentiation of the AHPCs significantly. Furthermore, strong evidence was demonstrated that the 3-D patterned surface influences astrocyte morphology and provides guidance cues for inducing neuronal differentiation that astrocytes alone cannot provide. The synergistic combination of the spatial control provided by the laminin-coated micropatterned substrate and the biological influence of the astrocytes led to selective neuronal differentiation of AHPCs [1].
Integrins, ECM molecules, and cell adhesion molecules, have important roles in CNS development inducing, coordinating, and regulating many complex cellular processes including cell growth, differentiation, migration, survival, proliferation, tissue organization and matrix remodeling [6-9]. It has been observed in vitro and in vivo that various ECM components regulate the differentiation of NSCs [10-12]. Our previous results demonstrated that the interaction between the micropatterned surface and the presence of the longitudinally aligned astrocytes effectively promoted oriented growth and instructed significant neuronal differentiation among the adherent NPCs [1]. We proposed that in this multi-dimensional environment, the aligned astrocytes present discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both. These factors may include specific molecules, originating from the extracellular microenvironment, known to mediate cellular proliferation, differentiation, process outgrowth and adhesion of neurons to astrocytes [13-15]. Localization of the soluble factors released by astrocytes in the microgrooves could have potentially influenced AHPC differentiation. It has been demonstrated that multiple interactions with the microenvironment, or ‘niche’, regulate NPC fate [12]. It is possible that the stem cell niche has been mimicked in vitro through the presentation of an optimal combination of signals necessary for neuronal differentiation to the AHPCs by the micropatterned PS-LAM environment.

Contact between AHPCs and astrocytes might also lead to competition between the different cells types for the same diffusible factors. In the present study, we are investigating further the astrocyte-derived factors in the multi-dimensional environment described above with a non-contact co-culture system utilizing a Transwell® semi-porous membrane insert to separate the astrocytes from AHPCs cultured on micropatterned substrates in the same well. In this non-contact experimental setup, factors released by the astrocytes are freely available to the AHPCs, with very limited, if any, localization of the factors within the microgrooves. In an effort to identify the optimal combination of signals creating both biological and spatial control over AHPC differentiation, we have examined
whether the factors responsible for this selective differentiation in the co-cultures were contact-mediated or soluble or both and possible mechanisms behind this interaction of guidance cues.

5.3 Materials and Methods

5.3.1 Micropatterned substrate fabrication

Polystyrene (PS) was chosen for substrate fabrication, as it is a biocompatible polymer that is used extensively in cell culture experimentation. Conventional photolithographic techniques and reactive ion etching were used to fabricate silicon wafers with the desired micropatterns that were then transferred to the polymer substrates using solvent casting [16]. The patterns used were described by: groove width (µm)/groove spacing (or mesa width) (µm)/groove depth (µm). To study the physical guidance of AHPCs on micropatterned PS substrates, the pattern dimensions used for these experiments were 16/13/4 µm, which are the same as the pattern dimensions used in the contact co-culture experiments [1].

The solvent cast polymer substrates were fabricated from an 8% (w/v) polystyrene (MW 125,000-250,000) (Polysciences, Inc., Warrington, PA) solution in toluene. Substrate thicknesses of approximately 50-70 µm were achieved using solvent casting techniques. After casting and drying for a minimum of 24 hours, the PS substrate was removed by soaking in deionized (DI) water and then sterilized with 70% ethanol. The micropatterned substrates were imaged using scanning electron microscopy (SEM). After mounting, the samples were sputter coated (SEM Coating Unit E5100, Polaron Instruments, Inc., Watford Hertfordshire, UK) with gold and imaged using a JEOL JSM-840A at an accelerating voltage of 20 kV, a 50 µm diameter aperture and a vacuum level of 1 x 10^{-6} Torr.

Cell growth chambers were constructed using PTFE (Teflon®) o-rings (Small Parts, Inc., Miami Lakes, FL), glass coverslips, and PS substrates (1 cm² in area) as described previously [16]. The PS substrates were coated with poly-L-lysine (PLL; Sigma, St. Louis, MO) solution at a
concentration of 100 μg/ml and laminin (LAM, Sigma) at a concentration of 10 μg/ml in Earle's Balanced Salt Solution (EBSS; Gibco, Grand Island, NY).

5.3.2 **Astroglial cell isolation and purification**

All animal procedures were conducted in accordance with and had the approval of the Iowa State University Committee on Animal Care. A population of purified cortical astrocytes was obtained from neonatal rat pups as described in Recknor et al.[16] Briefly, cerebral hemispheres were freshly dissected from 1-3 day old Sprague-Dawley rat pups and treated with papain solution (20 IU/ml; 37 °C, 5% CO₂/95% air, 1 hour) (Sigma). After subsequent treatment with trypsin inhibitor solution (10 mg/ml; Sigma), the tissue was mechanically dissociated in modified minimal essential culture medium (MMEM). The cultures were grown to confluence in 25 cm² tissue culture flasks (T-25; Falcon) at 37 °C in a humidified 5% CO₂/95% air atmosphere. The culture medium, MMEM, consisted of minimum essential medium (MEM; Gibco) supplemented with 40 mM glucose, 2 mM L-glutamine, 1 mM pyruvate and 14 mM NaHCO₃, penicillin (100 IU/ml) and streptomycin (100 μg/ml) with 10% v/v fetal bovine serum (FBS; HyClone, Logan, UT), pH 7.35.

Enriched type-1 astrocyte cultures were prepared as previously described [16]. After the cultures reached confluency (~8 days), the cells were shaken twice on a horizontal shaker at 260 RPM at 37 °C, first for 1.5 hours and then for 18 hours. The remaining adherent cells were enzymatically detached with trypsin (0.1% in EBSS; Sigma), pelleted (100 X g, 10 min.), resuspended in MMEM and passaged into 25 cm² tissue culture flasks. Cultures were fed every 3 days and were not passaged more than 8 times. Over 90% of the cells cultured under these conditions were selectively labeled by GFAP antibody (data not shown) confirming their astrocyte identity.
5.3.3 Adult hippocampal progenitor cell culture

Adult rat hippocampal progenitor cells (AHPCs) were originally isolated from the brains of adult Fischer 344 rats as reported by Palmer and colleagues [17]. The expanded cultures of single clones were infected with retrovirus to express enhanced GFP [17, 18]. The AHPCs were maintained in plastic tissue culture flasks (T-75, Fisher Scientific, Pittsburgh, PA) coated with poly-L-ornithine (10 μg/ml; Sigma) and mouse-derived laminin (5 μg/ml; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4). The AHPCs were maintained in complete medium containing Dulbecco’s modified Eagle’s medium/Ham’s F12 (DMEM/F12, 1:1; Omega Scientific, Tarzana, CA) supplemented with N2 (Gibco BRL, Gaithersburg, MD), 20 ng/ml basic fibroblast growth factor (human recombinant bFGF; Promega Corporation, Madison, WI), and L-glutamine (2.5 mM L-glu; Gibco BRL, Gaithersburg, MD). For *in vitro* analysis on laminin-coated polystyrene (PS-LAM) substrates, the AHPCs were detached from the T-75 flask using ATV solution (Gibco BRL, Gaithersburg, MD), harvested and collected by centrifugation at 1000g for 5 minutes. The pellets were resuspended in the culture medium stated above without FGF (referred to as differentiation medium) or co-culture medium (described below) and triturated gently. The cells were then plated on the micropatterned PS substrates coated with PLL (100 μg/ml in borate buffer) and laminin (10 μg/ml EBSS) (PS-LAM substrates) at initial densities of 10,000 to 15,000 cells per cm². Cells were maintained at 37 °C in a 5% CO₂/95% air atmosphere for 6 days in culture medium.

5.3.4 Co-culture of astrocytes and AHPCs

For contact co-cultures, purified astrocytes were plated onto PS-LAM substrates inside growth chambers and cultured for 2 days to generate near confluent monolayers. AHPCs were plated on top of the astrocyte monolayer at approximately 15,000 cells per cm². The co-cultures were maintained in a mixed medium that consisted of astrocyte MMEM (without FBS) in a 1:1 mixture
with AHPC differentiation media (referred to as co-culture media). As controls, AHPCs and astrocytes were plated in the same co-culture medium at the same density. AHPC-astrocyte co-cultures, AHPCs and astrocytes were grown for 6 days and then fixed with 4% paraformaldehyde in 0.1 M PO₄ buffer (pH = 7.4). Within 6 days, under the co-culture conditions, most of the astrocytes were immunoreactive for glial fibrillary acidic protein (GFAP, see Antibodies below) with little contamination by cells immunoreactive for TuJ1 (an antibody directed against class III β-tubulin), Map2ab (an antibody directed against microtubule associated protein 2) or RIP (for identification of terminally differentiated oligodendrocytes).

For non-contact co-cultures, purified astrocytes were plated onto the 0.2 μm semi-porous polyester membrane of Transwell® inserts inside 6-well plates (Corning, Inc., Corning, NY) (Figure 1) and cultured for 2 days in MEM. Astrocyte seeding densities were calculated and equally approximated across conditions taking into account the surface area of the insert and PS-LAM substrate used for contact co-cultures. After 2 days, the MEM was removed from the inserts and the astrocytes and wells of the 6-well plates were rinsed thoroughly with EBSS. The media inside the inserts was replaced with co-culture medium and the astrocytes were incubated in this medium for 4 hours. During this time, AHPCs were plated on PS-LAM substrates in co-culture medium at approximately 30,000 cells/ml. The AHPCs were allowed to attach for 2 hours before removing the o-ring containing the cell suspension. The substrates were then placed inside the same well (of the 6-well plates) as the inserts without contact and additional co-culture medium was added to the wells. AHPC-astrocyte non-contact co-cultures were grown for 6 days. Then, the astrocytes (on the insert membranes) and the AHPCs (on the PS-LAM substrates) were fixed with 4% paraformaldehyde in 0.1 M PO₄ buffer (pH = 7.4). Within 6 days, under the co-culture conditions, most of the astrocytes were immunoreactive for GFAP (see Antibodies below). As a control, AHPCs were also plated in astrocyte conditioned co-culture medium (ACCM). Purified astrocytes were cultured in tissue culture treated Petri dishes (35-mm, Fisher Scientific, Pittsburgh, PA) in MEM at similar seeding densities
to those plated on the 0.2 μm polyester membranes of the Transwell® inserts. After 2 days, the astrocytes were rinsed thoroughly with EBSS and the MMEM was replaced with co-culture medium. The same day that the AHPCs were plated for the co-cultures, AHPCs were also plated onto PS-LAM substrates in fresh co-culture medium for the controls. After the AHPCs and astrocytes had been cultured for 2 days in co-culture medium, ACCM was removed from the astrocytes cultured in the Petri dishes and centrifuged. The supernatant was then used to replace media removed from the AHPCs growing on the PS-LAM substrates. Media replacement was performed every 24 hours.

5.3.5 Analysis of AHPCs In Vitro: Immunocytochemistry

Cells cultured on micropatterned/non-patterned PS-LAM substrate surfaces were processed for immunocytochemistry according to standard protocols described previously [16]. Briefly, cells were first rinsed in 0.1 M PO₄ buffer, fixed using 4% paraformaldehyde in 0.1 M PO₄ buffer, rinsed, and processed. Cultured cells were incubated in blocking solution (3-5% donkey serum, 0.4% bovine serum albumin (BSA; Sigma), and 0.2% TritonX-100 (Fisher Scientific)) for 45 minutes. Specific primary antibodies (see Antibodies section) were used to identify differentiated neurons and glia. Cells were incubated in primary antibodies overnight at 4°C in a humid chamber, washed in potassium PBS (KPBS, 0.15 M NaCl, 0.034 K₂HPO₄, 0.017 KH₂PO₄, pH 7.4) with Triton-X, incubated in the appropriate biotinylated secondary antibodies for 2 hours, rinsed, and incubated with streptavidin Cy3 (Jackson ImmunoResearch, West Grove, PA) in the dark for 30 minutes. The cells were then rinsed and stained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI), a semi-permeant nucleic acid stain. DAPI was diluted at 1:100 in PBS and applied for 15 minutes. Preparations were rinsed and then mounted onto microscope slides using an antifade mounting medium (Gel Mount; Biomedica Corp., Foster City, CA).

Cells were observed using light microscopy (Olympus IMT-2 bright field/phase contrast microscope) and epifluorescence microscopy (Nikon Corp., Melville, NY) during culture. Digital
images were taken throughout experimentation using a Nikon Eclipse (Nikon Corp.) inverted microscope equipped with standard epifluorescence illumination and differential interference contrast (DIC) optics equipped with a cooled digital camera (ORCA, Hamamatsu) controlled by MetaMorph software (Universal Imaging Corporation, West Chester, PA). Cultured AHPCs were also examined using a photomicroscope (Microphot FXA; Nikon Corp.). Images were captured with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a frame grabber (Megagrabber; Perceptics, Knoxville, TN, in a Macintosh computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

5.3.6 Quantitative analysis of immunocytochemistry

Following immunocytochemical procedures on PS-LAM substrates, the preparations were examined and photographed using fluorescence microscopy (Nikon epifluorescence microscope with Hamamatsu digital camera). A 20X objective was used to examine twelve microscope fields, each field representing 0.24 mm² (546 μm by 438 μm). Six fields were examined from the micropatterned half and non-patterned half of the PS-LAM substrates. The following counts were made in each field: the total number of cells (DAPI stained nuclei), the number of GFP⁺ cells and the number of cells expressing TuJ1. These data were used to calculate the percentage of cells labeled with DAPI and with one of the antibody markers (mentioned above) on each PS-LAM substrate. The data collected after 6 days for each antibody was compared and analyzed. The experiment was repeated three to four times with two substrates per treatment in each experiment.

5.3.7 Antibodies

Differentiated neurons were identified using an antibody directed against class III β-tubulin (TuJ1, mouse IgG, Research Diagnostics, Inc.) diluted at 1:750. Anti-glial fibrillary acidic protein
(Anti-GFAP, mouse IgG; ICN) diluted at 1:600 was used as a marker of astrocytes. Terminally differentiated oligodendrocytes were identified using Anti-RIP (1:1600) (mouse IgG, obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Biology, University of Iowa, under contract NO1-HD-2-3144 from the NICHD). Biotinylated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) was used at a dilution of 1:500. All primary and secondary antibodies were diluted in blocking solution. Streptavidin Cy3 was diluted in PBS to 1:15,000. Negative controls were performed in parallel by omission of the primary and secondary antibodies. No antibody labeling was observed in the controls.

5.3.8 Statistical analyses

Statistical analyses were performed on the percentage of cells expressing the antibody of interest. Six regions (or fields) were examined from the micropatterned halves and non-patterned halves of the PS-LAM substrates. Since each region was a subsample of that half of the substrate, the means of the regions were calculated for use in the analysis so that each region would receive equal weight. The experiment followed a split plot design since the treatment (co-culture or control) was applied to the entire substrate and both the micropatterned and non-patterned regions were within one substrate. Due to this design, there were two random effects in the model, (1) for the whole plot effect, consisting of the error term for the treatment and (2) for the split plot effect, which included the pattern effect and the interaction between the treatment and pattern. The whole plot experimental unit was the entire PS-LAM substrate (approximately 1 cm² in area) while the split plot experimental unit was the half of the substrate that was either patterned or non-patterned. For TuJ1, n=12 for the whole plot analysis and n=24 for the split plot analysis. Mixed model analysis was performed on the means using the PROC MIXED procedure in SAS. All tests performed were two-sided tests and p-values less than an alpha value of 0.05 were considered significant. Analysis of the residuals was performed and there was no evidence of assumption violations.
5.4 Results and Discussion

Cells were cultured on square substrates having 0.5 cm$^2$ of patterned substrate (Figure 2) adjacent to 0.5 cm$^2$ of non-patterned substrate (as a control). To study the physical guidance of AHPCs on micropatterned PS substrates, the pattern dimensions optimized for these experiments were 16/13/4 μm (groove width/ groove spacing (or mesa width)/groove depth), which were based on previous studies involving the alignment of astrocytes and AHPCs [1, 16, 19]. In synergy with the physical guidance of the micropatterned PS substrate, chemical and biological cues were incorporated into a non-contact co-culture system to further investigate the astrocyte-derived factors involved in the directed outgrowth and selective differentiation of AHPCs in the contact co-culture system previously investigated [1]. This non-contact co-culture system was devised utilizing a Transwell® semi-porous membrane insert to separate the astrocytes from AHPCs cultured on micropatterned substrates in the same well. The soluble and/or contact mediated factors controlling AHPC outgrowth and differentiation were investigated as well as possible mechanisms behind this interaction of guidance cues.

5.4.1 Effect of guidance cues on AHPC outgrowth and differentiation

AHPCs were morphologically and immunocytochemically characterized on the micropatterned/non-patterned PS-LAM substrates in non-contact co-cultures at 6 days in vitro using cell type specific antibodies. The phenotypes of AHPCs growing under differentiation conditions in co-culture media were assessed using antibodies directed against class III β-tubulin (TuJ1), a protein characteristic of early neurons and the glial markers, receptor interacting protein (RIP) and glial fibrillary acidic protein (GFAP), for oligodendrocytes and astrocytes, respectively. Counterstaining with DAPI allowed visualization of the nuclei. The percentages of AHPCs immunoreactive (IR) for TuJ1, RIP and GFAP in co-culture, non-contact and contact, and in the absence of astrocytes on the patterned and non-patterned sides of the PS-LAM substrates are displayed in Table 1.
After six days in vitro, a significantly greater proportion of the AHPCs in non-contact co-culture were TuJ1-IR on the micropatterned (72.3%) and planar substrate (66.7%) compared to contact co-cultures on the micropatterned (34.5%) and planar substrate (20.9%) and AHPCs in the absence of astrocytes (α = 0.05; p < 0.0001). In addition, the results showed that, in contact co-cultures, on the planar surfaces, while approximately 20% of the AHPCs were TuJ1-IR in the co-cultures and approximately 16% were TuJ1-IR in the absence of astrocytes, there was no evidence of a significant difference between the two conditions (α = 0.05; p = 0.56). However, on the micropatterned surfaces, the percentage of TuJ1-IR AHPCs in the co-cultures (34.5%) was significantly higher than that in the absence of astrocytes (15.7%) (α = 0.05; p = 0.001). It was apparent that in the contact co-cultures both AHPC morphology and phenotype were influenced by the 3-D topography of the patterned substrate as well as the presence of the aligned astrocytes. However, in the non-contact co-cultures, even though the presence of the astrocytes contributed to a significant increase in percentage of TUJ-IR cells, there was not a significant difference between TuJ1 immunoreactivity of the AHPCs on the micropatterned surfaces as compared to the planar surfaces. In the astrocyte conditioned co-culture media condition, approximately 15% of the AHPCs were TuJ1-IR with no apparent differences in immunoreactivity between the micropatterned and non-patterned surfaces. As many soluble factors are short-lived, it is possible that the astrocyte conditioned media was not being replaced often enough to affect the AHPCs in the same manner as in the non-contact co-cultures.

AHPCs in the non-contact co-culture on the micropatterned surfaces appeared more elongated than those in the contact co-cultures (and significantly more so than those in the absence of astrocytes). On the patterned and planar surfaces, these cells were more robust having longer processes and larger cell bodies (Figure 3). In these co-cultures, directed neuritic extension was observed on the micropatterned surfaces with AHPCs extending long, elaborate TuJ1-IR processes in the direction of the grooves (Figure 3a-c). In the non-contact cultures, processes of TuJ1-IR AHPCs
were significantly longer than those observed in the contact co-cultures or in the absence of astrocytes. However, further characterization needs to be done regarding elongation (i.e. length measurements) and comparisons need to be made among culture conditions to ensure statistical significance of this observation.

After six days in vitro, similar proportions of AHPCs in the non-contact co-culture expressed RIP on either the patterned (19.5%) or non-patterned (25.3%) surfaces compared to those cells in contact co-cultures on the either the patterned (23.8%) or non-patterned (22.6%) surfaces. There was no evidence of a significant difference between the percentage of RIP-IR AHPCs on either patterned or non-patterned surfaces or non-contact and contact co-cultures or AHPCs in the absence of astrocytes ($\alpha = 0.05; p = 0.50$, $p = 0.97$ and $p = 0.66$, respectively). In non-contact co-cultures, on the micropatterned substrates, RIP-IR cells elaborated extensive processes weaving intricately inside the grooves and along the mesas of the substrate. On the non-patterned substrates, radial outgrowth from RIP-IR cells was observed. GFAP-IR cells displaying flattened morphologies with large nuclei were rare in all conditions examined and composed less than 0.1% of immunoreactive cells.

5.4.2 Microenvironmental regulation of AHPC outgrowth and differentiation

There are multiple factors in the microenvironment directly surrounding cells that can induce and maintain their functional stability. Elucidating which factors are involved and how they interact with cells is a principal aim of tissue engineering. Cellular micropatterning has been used to study the effects of the microenvironment on cell growth and differentiation. Microfabrication technology has been applied to the design of substrates having specific architectures for such purposes [20]. Culture environments incorporating these substrates are designed to encourage isolated cells to function as they would in their in vivo microenvironments or niches. In our contact co-culture studies with astrocytes and AHPCs, we discovered that the contact co-culture environment on PS-LAM substrates provided control over neuronal differentiation and neurite alignment on topographically
different regions of the same substrate. While the exact mechanism was not deduced from this study, we proposed that the aligned astrocytes presented discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both.

To further investigate the astrocyte-derived factors in the multi-dimensional environment described above, a non-contact co-culture system was studied. It was determined that astrocyte-derived soluble factors can induce neuronal differentiation of the AHPCs. With significantly more cells expressing TuJ1 in the non-contact co-culture system than in the contact co-culture system, it appears that soluble cues may have a stronger effect on neuronal differentiation compared to the contact mediated factors or combination of soluble and contact mediated factors that were presented by aligned astrocytes in the contact co-culture. We propose that, in the contact co-culture, the astrocytes may be inhibiting neuronal differentiation to some extent. It is conceivable that the AHPCs are in competition with the astrocytes in the contact cultures for the released soluble factors. Furthermore, there may be constrained diffusion of the factors involved. In the contact co-culture conditions, if the astrocyte monolayer is considered the point source of soluble or membrane-associated factors, direct cell-cell interactions within the monolayer itself may be hindering the diffusion of soluble factors into the media and the temporal and spatial presentation of these factors to the AHPCs. However, in the non-contact co-cultures, diffusion is not constrained, having a major effect on the presentation of soluble cues to the AHPCs. Astrocyte-derived soluble factors are being released directly into the media and are presented to the AHPCs immediately upon release. As many of these soluble factors are known to be short-lived, as confirmed by the astrocyte conditioned co-culture media experiments, it is important for these soluble cues to be distributed in a relatively short period of time to the AHPCs for maximum effect on outgrowth and differentiation.

Even though the presence of the astrocytes contributed to a significant increase in percentage of TUJ-IR cells, there was not a significant difference between TuJ1 expression on the micropatterned surfaces as compared to the planar surfaces. However, in the non-contact co-culture system, enhanced
neurite outgrowth was observed, and elongated neurites were oriented in the direction of the grooves on the micropatterned surfaces. In the contact co-cultures, the significant difference seen among AHPCs in co-culture and in the absence of astrocytes on the patterned surfaces and not on the planar surfaces suggested that the 3-D environment provided a means for concentrating factors necessary for inducing neuronal differentiation. We proposed that microgradients of such molecules may have been established as the astrocytes aligned and released soluble factors that bind to components of the ECM deposited in the grooves and on the mesas of the micropatterned surface. As the AHPCs in the contact co-culture interacted with the astrocytes in this 3-D microenvironment, enhanced neuronal differentiation among the AHPCs may have resulted from these localized microgradients. However, in the non-contact co-culture, it is probable that no such gradients were set up as the astrocyte-released factors were uniformly available to all AHPCs on the substrates, whether or not the surface was micropatterned. Since there was not direct competition with the astrocytes for the released factors, the AHPCs are directly and immediately affected by these soluble cues. These factors may concentrate within the grooves but they are presumably taken up by the AHPCs that they come into contact with immediately, before they can accumulate, since many of the factors are short-lived. This concentration of soluble cues served to increase directed neurite outgrowth and elongation of the TuJ1-IR AHPCs, which in combination with the 3-D topography results in elaborate, oriented neurites on the micropatterned surfaces. Therefore, it appears that neurite outgrowth and neuronal differentiation in contact co-cultures may be constrained diffusion (microgradient)-controlled while it is primarily bulk diffusion-controlled in the non-contact co-cultures. It is well known that extracellular transport processes play critical roles in cellular morphogenesis [21]. Gradients of morphogens, or substances that assign different cell fates at different concentrations, specify many patterns of cell and tissue organization during development. Gradients form by morphogen transport from a localized site, but whether this occurs by simple diffusion or by more elaborate mechanisms is unclear [22]. It not apparent precisely how diffusion may be controlling outgrowth and differentiation
in these co-culture systems. Further studies need to be undertaken to distinctly characterize the mechanism(s) behind this enhanced effect and to elucidate the specific soluble factor(s) involved.

5.5 Acknowledgments

Financial support from The Glaucoma Foundation to DSS and from the National Institutes of Health to SKM and DSS are gratefully acknowledged. The authors would like to thank Dr. Fred Gage at the Salk Institute for the gift of the AHPCs. The authors are also grateful to Dr. Robert Doyle at the Roy J. Carver Laboratory for Ultrahigh Resolution Biological Microscopy at Iowa State University (ISU) for his helpful advice and suggestions and Justin Recknor at ISU for his work on the experimental statistics. The authors would also like to thank Chris Blong for his help with substrate preparation.
Table Caption

Table 1. AHPC immunoreactivity under differentiation conditions

Figure Captions

Figure 1. The non-contact co-culture system utilizing a Transwell® semi-porous membrane insert to separate astrocytes (top well) from AHPCs (bottom well). Astrocyte monolayers were cultured on the insert membranes in the bottom well. Soluble factors are able to diffuse into the bottom well affecting the AHPCs immediately upon release.

Figure 2. A scanning electron microscopy image of a micropatterned polystyrene (PS) substrate with groove dimensions 16/13/4 μm (groove width/groove spacing/groove depth). Patterned substrates with these dimensions were used throughout this study. Scale bar = 10 μm.

Figure 3. Expression of class III β-tubulin (TuJ1) by AHPCs in co-culture with astrocytes-without contact- on micropatterned (a-c) and non-patterned (d-f) PS substrates. (a,c) GFP-expressing AHPCs and (b,d) TuJ1-IR cells. (c,f) Merged images of GFP-expressing (green) AHPCs labeled with TuJ1 (red). Dotted lines indicate the location of a groove on the micropatterned surface. Scale bar = 10 μm.
### Table 1. AHPC immunoreactivity under differentiation conditions

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<td>TuJ1</td>
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<td>AHPCs only</td>
<td>TuJ1</td>
<td>14.64 ± 2.26</td>
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<td>(in the absence of Astrocytes)</td>
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<td></td>
<td>GFAP</td>
<td>&lt; 0.1</td>
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^ Data are the percentage of immunoreactive cells mean ± s.e.m. and represent pooled data from at least three separate culture sessions. The expression of TuJ1 was significantly different between AHPCs in non-contact co-cultures, contact co-cultures, and AHPCs only (p < 0.0001) and in contact co-culture on patterned and non-patterned surfaces (p = 0.003) after 6 days.

* Significantly different from non-patterned surfaces.

+ Significantly different from contact co-cultures and AHPCs only on patterned and non-patterned surfaces.
Figure 1.

Astrocyte-AHPC Co-cultures across a membrane filter

- Inner chamber: Astrocytes
- Bottom chamber: AHPCs

Culture well

Insert

Astrocytes

AHPCs

Membrane filter

PS Substrate
Figure 2.
Figure 3.
5.6 References


CONCLUSIONS

Biomimetic strategies have been employed to study the effects of a synergistic combination of physical, chemical, and biological cues on the outgrowth of cell types specific to the central nervous system. Polystyrene (PS) was used for fabricating micropatterned solvent cast substrates. Conventional photolithographic techniques and reactive ion etching or deep reactive ion etching were used to prepare silicon wafers having desired micropatterns that were imprinted onto the PS substrates using solvent casting. Direct etching was also experimented with to create micropatterns on the PS substrates. As discussed in Chapter 3, in an effort to promote central nerve regeneration in vivo, directional growth of astrocytes has been achieved on polymer substrates in vitro manipulating a combination of physical and chemical guidance cues. Using a surface tension based technique for laminin application on the micropatterned substrates, more laminin was selectively adsorbed to the groove regions. The adsorbed laminin presented chemical cues for cell adhesion and spreading. Laminin-coated micropatterned substrates significantly promoted the directional guidance of growing astrocytes in vitro. The astrocytes extended filaments along the inside of the groove region of the PS substrates as well as on the mesas aligning to within 20° and 10° of the groove direction. Over 85% of the astrocytes aligned within 20° and 60% aligned within 10° of the groove direction on the laminin-coated PS substrate. The combination of the topographical and chemical cues provided the maximum effect on astrocyte outgrowth and alignment.

As the next step in generating a supportive environment for the directed outgrowth and selective differentiation of neural stem/progenitor cells, the effect of physical and chemical cues on the differentiation and outgrowth of the adult hippocampal progenitor cells (AHPCs) was explored. Micropatterned PS substrates were chemically modified with poly-L-lysine and laminin (PS-LAM) contributing substrate-associated factors for adhesion, differentiation and directed outgrowth. AHPCs
cultured on the micropatterned PS-LAM substrates were oriented in the groove direction exhibiting over 75% AHPC alignment. There was not a significant difference in the percentage of cells expressing TuJ1, RIP, or GFAP on micropatterned surfaces as compared to planar surfaces. In an effort to optimize the AHPC microenvironment, AHPCs were co-cultured on top of astrocyte monolayers on the PS-LAM substrates. As presented in Chapter 4, the physical cues, in synergy with chemical and biological guidance cues, were found to direct outgrowth and facilitate selective neuronal differentiation of AHPCs. After six days, many AHPCs acquired neuronal morphology and significantly more cells expressed TuJ1 in co-culture on micropatterned surfaces than on planar surfaces or growing in the absence of astrocytes. This indicates that substrate 3-D topography, in synergy with chemical (laminin) and biological (astrocytes) facilitates neuronal differentiation of the AHPCs. Through multi-dimensional cell-cell interactions, this integrated co-culture environment provided biological and spatial control to the adherent NPCs. The distribution of active neurite outgrowth-promoting molecules presented by the aligned astrocytes on the micropatterned PS-LAM substrates created a multi-dimensional microenvironment instructing neuronal fate commitment and promoting oriented neurite outgrowth on topographically different regions of the same substrate. We proposed that the aligned astrocytes presented discrete guidance cues to the overlying AHPCs involving contact mediated or soluble factors or a combination of both.

As presented in Chapter 5, to further investigate the astrocyte-derived factors in the multi-dimensional environment described above, a non-contact co-culture system was devised. This system utilized a Transwell® semi-porous membrane insert to separate the astrocytes from AHPCs cultured on micropatterned substrates in the same well. In an effort to identify the signals controlling AHPC outgrowth and differentiation, the soluble and/or contact mediated factors involved were investigated as well as possible mechanisms behind this interaction of guidance cues. From these experiments, it was determined that astrocyte-derived soluble factors can induce neuronal differentiation of the AHPCs with significantly more cells expressing TuJ1 in the non-contact co-culture system than in the
contact co-culture system. In the non-contact co-culture system, enhanced neurite outgrowth was also observed, and elongated neurites were oriented in the direction of the grooves on the micropatterned surfaces. Therefore, it appears that soluble cues may have a stronger influence on neuronal differentiation and neuritic extension compared to the contact mediated factors or the combination of soluble and contact mediated factors that were presented by the monolayer of aligned astrocytes. The results also point to the potential role of localized concentration of these factors within the microgrooves as a reason for the differences in differentiation on micropatterned and planar substrates in the contact as opposed to the non-contact co-cultures. This integration of multiple cues is important for understanding and controlling NSC differentiation and in designing scaffolds or conduits for guided nerve regeneration \textit{in vivo} within the CNS.

Combining physical, chemical and biological guidance cues that enable spatial control over AHPC differentiation on topographically different regions of the same substrate can potentially generate a supportive environment for eliciting regeneration and restoring function in the injured or diseased CNS. Integrating multiple stimuli to direct the lineage of endogenous or engrafted CNS-derived precursor cells on specific substrate regions presents opportunities to mimic the natural \textit{in vivo} environment and elucidate the mechanisms behind efficient stem cell - mediated repair of the CNS. This research provides insights into mechanisms of NSC differentiation and a foundation for a promising regeneration strategy for guided CNS repair. To achieve the desired morphological and functional characteristics essential for neural regeneration, an integrative approach, employing NPCs with the proper biological, biochemical and biophysical signaling, such as the multiple stimuli presented in this research, is necessary. The dynamic microenvironment that results from this synergistic combination of guidance cues could be applied as a tissue engineering strategy in which cells and signaling molecules are combined first \textit{in vitro}, to create a biomaterial suitable for implantation. Scaffolds integrating the physical guidance effects of the micropatterned polymer substrates with chemical guidance mechanisms and the biological influence of astrocytes to direct
NSC differentiation and outgrowth provide a microenvironment that can potentially support regeneration of the diseased or injured optic nerve \textit{in vivo}. Furthermore, such a synergistic combination of guidance cues can be applied \textit{in vivo} strategies for facilitating regeneration in other regions of the CNS as well.
CHAPTER 7

FUTURE DIRECTIONS

Creating an environment that incorporates multiple guidance cues to direct and selectively instruct the terminal lineage of endogenous or engrafted NSCs on topographically different regions of the same substrate, as was achieved in this dissertation, offers exciting opportunities for elucidating the mechanisms behind cellular differentiation, nerve regeneration and specific considerations for the efficient stem cell-mediated repair of the central nervous system (CNS). Gaining insights into the biology of NSCs through control over outgrowth and differentiation will provide useful information for in vitro and in vivo experimentation into the regeneration and repair of the CNS. The information gained from this work has direct implications for guided nerve regeneration in vivo within the CNS.

Since this research has focused on determining the optimal combination of signals controlling AHPC outgrowth and differentiation, a direct extension of this work is to determine the exact mechanism(s) behind the interaction of guidance cues in the contact and non-contact co-culture systems and to elucidate the specific soluble factor(s) that are involved in the directed outgrowth and selective neuronal differentiation of the AHPCs. Using these cues and the co-culture systems developed, directed and enhanced AHPC differentiation, outgrowth, and alignment in vitro can continue to be investigated using additional techniques such as gene expression analysis. Gene array analysis can be performed to study the up/down regulation of genes associated with neurogenesis. Reverse transcription polymerase chain reaction (RT-PCR) techniques can verify this analysis and be used to analyze and compare gene product concentrations on topographically different regions of the PS substrates for gene expression consistent with proteins characteristic of neurons. To determine the soluble factor(s) involved, in the non-contact studies, the conditioned medium could be tested for various factors using chromatographical techniques. As glia-derived cholesterol has recently been shown to mediate synaptogenesis in CNS neurons [1-3], cholesterol may be a factor that could be
investigated using size exclusion chromatography (SEC) or high pressure liquid chromatography (HPLC).

Further extensions of this work could also involve characterizing the AHPCs in the contact and non-contact co-culture systems physiologically to determine if the AHPCs are differentiating into functional neurons as well as taking the co-culture experiments out to longer time points. This research would include immunocytochemical analysis involving pre- and post-synaptic proteins as well as more mature neuronal and oligodendrocyte markers. It is conceivable that over time periods longer than one week, the TuJ1-IR AHPCs will become more mature, potentially develop synapses and presynaptic and postsynaptic differentiation among the neuronal cells may be enhanced by the astrocytes or astrocyte-derived factors. A valuable direction based on this work is in exploratory mathematical modeling efforts to study NSC migration and differentiation and the role of ECM proteins and diffusible factors. An understanding of cues that influence cell differentiation, positioning and alignment is important not only in tissue engineering but is critical in studying such areas as pattern formation in developmental biology. Finally, since this work focused strictly on adult rat hippocampal progenitor cells, another direct extension of this work is to examine a human line of NPCs to determine if the same enhanced neuronal differentiation can be replicated using the synergistic combination of guidance cues optimized in this dissertation. The work in this dissertation and potentially in the above research studies provide valuable fundamental knowledge into the mechanisms behind NSC outgrowth, migration and differentiation and enable the development of protocols for effective engineering strategies for incorporation of these cells into conduits to promote nerve regeneration in the CNS.

There will also be further investigation into the effect of localized electrical stimulation using microelectrodes arrays in conjunction with the micropatterned substrates to control the growth, migration and differentiation of AHPCs. The cellular mechanisms of nerve repair can be explored by creating an interface between biological and non-biological systems using microelectronics.
Microfabrication techniques are being used to develop a micropatterned PS conduit with embedded arrays of microelectrodes that can serve as electrical cues in the directed growth of regenerating axons. Cells can be cultured directly onto this substrate for in vitro experimentation. The microelectrodes can be used to stimulate nerve regeneration as well as for selectively recording electrical signals from axons observed in particular grooves and specific locations within the grooves of the micropatterned substrates. The cells in the grooves can be selectively stimulated electrically through gold electrodes throughout the length of the grooves (Figure 7.1). The advantage of this setup is that it allows certain cells located on the patterned surfaces to be selectively electrically stimulated, while other cells on the same substrate do not receive the electrical stimulation. This device also allows for selectively recording from individual cells in the grooves. The effect of this selective electrical stimulation in combination with physical, chemical and biological cues on the differentiation of AHPCs can also be investigated. Using the information gathered from such a device, an interface between optic nerve axons and retinal prosthesis can be pursued. This approach could provide a system that can eventually be used for the transmission of electrical signals from microelectronic devices, such as a retinal chip, through a bioartificial optic nerve to the brain where these signals are decoded and interpreted as visual images. With such a system, greater understanding and control of CNS function and regeneration can be accomplished.
Figure 7.1: The proposed first generation design for an array of microelectrodes embedded into a micropatterned PS substrate. Microfabrication techniques were used to develop this device. With such a device, an interface between optic nerve axons and retinal prosthesis can potentially be created.
References


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