CONTROLLING GROWTH CONE BEHAVIOR THROUGH SUBSTRATE PATTERNING.

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ABSTRACT

Throughout the process of development, billions of neuronal axons are responsible for navigating through the nervous system and synapsing onto their appropriate targets. To establish their individual paths, neurons are guided by a complex and dynamic map of biochemical, topographic, and mechanical signals. At the tip of each neurite lies the growth cone – a dynamic structure responsible for interpreting extracellular cues and steering neuronal growth in the appropriate direction. Growth cones in vivo exhibit various morphologies and behavioral changes, though the underlying mechanisms of these changes remain widely unknown. Through the use of substrate patterning techniques, we examined the relationship between growth cone morphology and behavior with the purpose of optimizing guidance in engineered regenerative systems.

We investigated multiple substrate patterning techniques in order to achieve easily observable manipulations to growth cone morphology. We found that common micro-contact printing and microfluidic techniques are poor at creating isolated regions of overlapping biochemical cues. We also found that established protein patterning methods for soft substrates...
are ill-suited for use on surfaces with nervous system-relevant elasticity. Using laser ablation, we
designed micron-scale patterns capable of confining dissociated mouse cerebellar granule
neuron growth cones to channels of different widths. Growth cone dynamics in these channels
were observed using time lapse microscopy. Growth cone area was decreased in channels
between 1.5 and 6 µm as compared to that in 12 µm and unpatterned substrates. Growth cone
aspect ratio was also affected as narrower channels forced growth cones into a narrow,
elongated shape. There was no difference in the overall rate of growth cone advance in uniform
channels between 1.5 and 12 µm as compared to growth on unpatterned substrates. The
percentage of time growth cones advanced, paused, and retracted was also similar. However,
growth cones responded to changes in confinement: growth cones in narrow lanes rapidly sped
up when encountering a wide region and then slowed down as they entered another narrow
region. Our results suggest that the rate of neurite extension is not affected by the degree of
confinement, but does respond to changes in confinement.

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1 BACKGROUND AND MOTIVATION

1.1 GENERAL INTRODUCTION

“If the brain were so simple that we could understand it, we would be so simple we couldn’t.” (Lyall Watson)

The human brain is an intricate, often unpredictable, and sometimes seemingly magical structure. In our mission to discover the secrets of how our brain works, most answers open up the doors for 10 more questions. The abnormal brain, whether stricken by disease or injury, presents an entirely different environment, and an entirely new set of questions. There are many ways to tackle these issues, but one thing is certain: if we see how a house is built, we will better understand how it functions, and will be more qualified to fix a burst pipe. With similar logic, we study the development of the nervous system to better understand function of the mature brain, as well as to better engineer regeneration and repair after injury.

To untangle the complexity of our nervous system, we investigate the birth, differentiation, and guidance of neuronal and glial cells. During embryonic development, the neuroectoderm gives rise to the neural tube and neural crest, which subsequently develop into the central and peripheral nervous system (Purves et al., 2004). Neural stem cells differentiate into neurons, the fundamental communication cell in the nervous system, and glia, the various supporting cells found adjacent to neurons. Development of the brain, spinal cord, and peripheral nervous system relies on the physical and chemical interactions between neurons, glia, and the extracellular matrix. Neurons send out one or more processes with the purpose of
seeking out and forming connections, or synapses, with their target cells. The process through which an axon finds its target is guided by multiple attractive and repulsive cues which are present on the surfaces of other cells, embedded in the extracellular matrix, or diffuse throughout certain areas in the form of biochemical gradients.

1.2 NEURONAL GUIDANCE AND THE GROWTH CONE IN VIVO

The tip of the neurite is characterized by a sensory and motile organ termed the growth cone. With fingerlike filopodia and web-like lamellipodia, growth cones guide developing neurons to their targets by sensing, integrating, and responding to a myriad of extracellular guidance cues. Active cytoskeletal processes in the growth cone selectively alter its structure in response to these cues, and dictate the formation of an axon or dendrite behind it. Following injury, regenerating neurons will again form growth cones in order to restore lost signaling pathways.

Santiago Ramón y Cajal, the first person to visualize growth cones, described them as a “sort of club or battering ram, endowed with exquisite chemical sensitivity, with rapid amoeboid movements, and with certain impulsive force, thanks to which it is able to proceed forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination” (Ramón y Cajal, 1909). Cajal’s intuition about the presence of a specifically guided pathway was challenged by Paul Weiss, who argued that nonspecific mechanical cues are responsible for organizing neurons into nerves and tracts (Weiss, 1934, 1945). Today, we know that both Cajal and Weiss were partially correct: growth cones have specific receptors which direct a chemotactic response to the environment, and are also sensitive to the mechanical and
topographic features of their surroundings. In vivo, an example of these mechanisms is evident in cortical development, where neurons follow a physical scaffold presented by radial glia while interpreting permissive and inhibitory cues which control the direction of neurite extension (Purves et al., 2004; Malatesta et al., 2008). In the cerebellum, the eponymous cerebellar granule neurons (CGNs) are polarized as a result of an extracellular chemical signal, and then migrate along Bergmann glial fibers (Gregory et al., 1988; Gupta et al., 2010). Developing ‘pioneer neurons’ establish both a topographic and biochemical pathway for other growth cones to follow. In the insect embryo, peripheral axonal pathways in the antennae, leg, and cerci are established by a pair of pioneer axons which are essential to the subsequent guidance of additional axons (Bate, 1976; Keshishian, 1980; Edwards et al., 1981; Klose & Bentley, 1989). Pioneer axons can fasciculate with each other, forming a scaffold on which subsequent axons will extend (Raper et al., 1983). The interplay between topographic fasciculation and chemotaxis is especially evident as axons extending on a common pathway diverge from one another at reproducible, biochemically patterned choice points, or ‘decision regions’, to join other specific axon bundle scaffolds (Raper et al., 1984). We propose that by understanding the dynamic morphology and behavior of growth cones in these decision regions, we can gain a better understanding of how to properly engineer neuronal regeneration in the central nervous system (CNS).

1.2.1 GROWTH CONES IN DECISION REGIONS

By studying the development of the visual system, researchers have discovered a particularly clear example of a growth cone ‘decision region,’ which is characterized by the
unique behavior of retinal ganglion cells (RGCs) in the optic chiasm. RGC somata are present in the retina, and growth cones extend through the optic nerve and into the optic chiasm. In the chiasm, growth cones either decussate across the midline and extend into the contralateral side of the brain, or continue along the ipsilateral pathway. Once they pass the optic chiasm, growth cones migrate along the optic tract and follow through to their respective targets, including the lateral geniculate nucleus or superior colliculus. The decussation seen in the chiasm is specific to the origin of the RGCs and serves to establish the circuitry responsible for binocular vision: axons from both eyes carrying information from the left visual field will end up in the right visual cortex, while those encoding the right visual field connect to the left visual cortex.

In mice, the developing optic chiasm is characterized by an aligned network of radial glia and a small population of early-born neurons (Mason & Sretavan, 1997). The chiasm is also home to a number of attractive and inhibitory guidance cues including Robo/Slit, Ephrins,
Semaphorins, Heparan and Chondroitin Sulfate Proteoglycans (CSPGs) (Table 1). These cues are necessary for proper growth cone steering through the chiasm. For example, enzymatic digestion of CSPGs with Chondroitinase-ABC significantly increased the number of axons crossing the midline and prevented ipsilateral extension (Chung, Taylor, et al., 2000). A similar effect is also seen when Ephrin-B2 function is blocked in vitro, preventing the inhibitory effect of the chiasm cells and eliminating ipsilateral projections from a semi-intact mouse visual system (Williams et al., 2003).

The ability to visualize neurons in vivo with the use of intracellular markers has allowed researchers to establish a link between the form, function, and position of RGC growth cones in the developing visual system. In the optic nerve and optic tract, growth cones tend to be small, exhibiting few filopodia and a streamlined shape, while growth cones in the medial optic chiasm tend to be large and complex with more visible filopodia and lamellipodia (Bovolenta & Mason, 1987). Furthermore, small, simple growth cones found in the optic nerve and tract tend to spend more time advancing, while complex growth cones in the optic chiasm are generally slower and spend a large portion of time paused (Mason & Wang, 1997). Growth cone advance is saltatory, characterized by a frequent switching between advances and pauses. While pausing occurs throughout almost all areas of the developing visual system, it is especially frequent in the midline of the chiasm. Furthermore, while pauses reflect a halt in forward activity, growth cones remain highly active with extensive filopodial and lamellipodial remodeling. (Mason & Erskine, 2000). This correlation between growth cone shape and behavior has been noted in other neuronal types and other areas in the nervous system, indicating that it is a fairly wide-spread phenomenon (Halloran & Kalil, 1994; Brittis & Silver, 1995).
There certainly exists an intimate relationship between growth cone morphology and behavior and the composition of the optic chiasm. First, growth cone expansion into more complex forms is likely related to an interaction with the perpendicular network of radial glia, as well as the overwhelming presence of biochemical guidance cues specific to the medial chiasm. In fact, ultrastructural studies in the developing chiasm have indicated that radial glia are virtually enveloped by complex growth cones (Godement et al., 1994; Marcus & Mason, 1995). Secondly, the pause in extension rate may be exhibited to essentially give growth cones enough time to properly integrate all of the available signals into an appropriate response. Therefore, the complex morphology exhibited by growth cones in decision regions may be indicative of an increased capacity to respond to extracellular cues. A complex growth cone exhibits more surface area and more filopodia, and complexity and filopodial movement is most noticeable before turning (Mason & Erskine, 2000). With these assumptions in mind, one may conclude that simple, streamlined growth cones must be less sensitive to guidance cues, either as a direct result of their morphology or due to a correlated factor such as surface receptor density. We propose that if growth cones are persuaded into a streamlined morphology, they may be more amenable to extend through an inhibitory glial scar following CNS injury.

1.3 INHIBITION IN THE GLIAL SCAR

Following pre- and post-natal development, very little axonal sprouting occurs in the adult CNS. Many biochemical pathways present in the embryonic stages of development are lost (Purves et al., 2004), and the mature nervous system relies heavily on plasticity, rather than the formation of entirely new pathways, to adapt to a changing environment. Therefore, the CNS is a
particularly poor candidate for regeneration following injury. Mild to medium-level injuries to the peripheral nervous system result in the clearance of dead and damaged cell debris by local Schwann cells, often followed by some appreciable level of neuronal regeneration (Scheib & Höke, 2013). Yet, with the exception of sensory projections in the olfactory bulb (Monti Graziadei et al., 1980) and a small hypothalamic pathway (Chauvet et al., 1998), myelinated axons in the CNS are nearly incapable of any endogenous functional regeneration (Li & Raisman, 1995). The lack of CNS axon growth after injury has been linked to an overwhelming inhibitory response from surrounding astrocytes, oligodendrocytes, and various biochemical cues, all of which form the glial scar.

The glial scar is a phenomenon specific to warm-blooded animals and is believed to serve as a mechanism to quickly repair the blood brain barrier following a breach of the CNS (Faulkner et al., 2004). An introduction of non-CNS molecules into the brain parenchyma is followed by an acute increase in macrophages and hypertrophic astrocytes in the injured area combined with a upregulation and release of CSPGs, therefore creating both a physical and biochemical barrier to regenerating axons (Preston et al., 2001; Silver & Miller, 2004). Severe injuries, such as those caused by a stab wound or projectile, also result in a breach of the meninges and subsequent infiltration of fibroblasts. In addition to the inhibitory CSPGs, necrotic oligodendrocytes break up to produce a number of inhibitory cues, including Nogo-A (Caroni & Schwab, 1988), OMgp (Kottis et al., 2002), semaphorin 4D (Schwab et al., 2005) and 5A (Goldberg et al., 2004), ephrin-B3 (Benson et al., 2005), and MAG (McKerracher et al., 1994). Therefore, a successful penetration of the glial scar by regeneration neurons requires a targeted approach to eliminate growth cone sensitivity to this complex inhibitory cocktail.
1.3.1 DYSTROPHIC GROWTH CONES

While examining injured axons in CNS tissue, Ramón y Cajal noticed a recurrence of enlarged, club-shaped, bulbous terminal ends that he deemed to be sterile (Clarke, 1992). More recent work with CNS regeneration has identified these unique structures as dystrophic growth cones, specifically occurring as a result of the inhibitory glial scar (Davies et al., 1999). Despite Cajal’s belief in the sterility of these structures, we know now that they are, in fact, prone to rehabilitation. Observations \textit{in vitro} as well as \textit{in vivo} have provided evidence that dystrophic growth cones have dynamic membrane veils and active endocytotic processes (Tom et al., 2004). Dystrophic growth cones can persist for months or years following injury, yet still remain active and capable of sprouting (Li & Raisman, 1995). \textit{In vitro}, manipulation of PKA or its downstream component paxillin, as well as inhibition of myosin II, resulted in dystrophic growth cone rescue and axon sprouting through a biochemical CSPG gradient (Hur et al., 2011; Kuboyama et al., 2013). \textit{In vivo}, regeneration of chronically injured neurons was achieved through transplantation into peripheral nerve grafts (Houle, 1991). Due to their endogenous presence and innate ability to regenerate, dystrophic growth cones are a likely target for therapeutic strategies in engineering CNS repair.

1.4 MECHANISMS OF GROWTH CONE GUIDANCE

To better understand neuronal development and to engineer techniques for functional recovery following injury to the nervous system, we must elucidate the mechanisms responsible for controlling growth cone behavior. Neuronal guidance through chemotaxis relies on extracellular signals acting directly on membrane-bound receptors, leading to the formation of
point contacts and a rearrangement of the growth cone cytoskeleton. While much progress has been made in studying these mechanisms, more research is necessary to improve therapeutic interventions following CNS injury.

1.4.1 CHEMOTAXIS

While capable of extending on plain, 2D surfaces, growth cones are known to use structural features, biochemical cues, and receptors to achieve guided directional movement through the nervous system. Over the past two decades, researchers have made spectacular progress in identifying various axon guidance molecules and their receptors. These molecules fall into several families, notably, the Netrins, Semaphorins, Slits, Ephrins, morphogens, neurotrophins, and extracellular adhesion molecules (Mortimer et al., 2008; Pollerberg et al., 2012). Certain molecules attract growth cones, while others repel them, and some are bifunctional. For example, the well-studied neurotrophin NGF will attract dorsal root ganglia (DRG) growth cones in culture, while Semaphorin 3A exerts the opposite effect, retarding axon extension and forcing growth cones to turn away (Joddar et al., 2013; McCormick et al., 2013). Netrin-1, on the other hand, acts bifunctionally in the developing floor plate, serving as an attractant to spinal cord commissural neurons and a repellant to trochlear motor axons (Colamarino & Tessier-Lavigne, 1995; Serafini et al., 1996).

Many ligand-specific receptors have been identified on the surface of the growth cone. The often-inhibitory activity of various Ephrins, for example, relies on Eph receptor signaling (Cutforth & Harrison, 2002; Giger et al., 2010), while the bifunctionality of the netrin-1 signal is controlled via the DCC and UNC receptor configuration (Chan et al., 1996; Wadsworth et al.,
The ligand-receptor interaction for CSPGs has remained a mystery until recently, when RPTPσ, a receptor previously shown to inhibit axon regeneration, had been found to regulate HSPG and CSPG signals (Aricescu et al., 2002; Thompson, 2003; Fox & Zinn, 2005; Johnson et al., 2006; Shen et al., 2009). It has been suggested that RPTPσ is also bifunctional, propagating signals from inhibitory CSPGs as well as permissive HSPGs through changes in clustering, but the mechanisms responsible for this behavior are currently under investigation (Coles et al., 2011).

1.4.2 POINT CONTACTS

During neurite extension, growth cones form miniature adhesions, called point contacts, between their membrane receptors and the surrounding environment. Similar to guidance cues, adhesion molecules provide an important roadmap during development (Kamiguchi, 2007; Myers et al., 2011; Pollerberg et al., 2012). Point contacts are initiated through integrins, cadherins, and other molecules embedded in the growth cone membrane, and may form bonds to neighboring cells, as well as proteins in the extracellular matrix (ECM). Cadherins, for example, interact homophilically with surface molecules expressed on adjacent cell membranes (Kolodkin & Tessier-Lavigne, 2011), while integrins bind to various proteins, including laminin, which are embedded in the ECM (Tomaselli et al., 1988). Inside the growth cone, integrin receptors involved in point contacts attract an assembly of multiple scaffolding proteins, including talin, paxillin, vinculin, FAK, and Src (Myers et al., 2011). Scaffolding proteins can bind directly to actin filaments, thus creating a structural link between an extracellular ligand and the growth cone’s cytoskeleton.
1.4.3 GROWTH CONE CYTOSKELETON

The growth cone cytoskeleton is directly responsible for any exhibited changes in morphology, and would therefore likely play a role in any link between morphology and behavior, whether seen in a decision region or an in vitro environment. The process of growth cone advance can be divided into three steps: protrusion, engorgement, and consolidation (Goldberg & Burmeister, 1986). These steps are defined by the relative movements of filamentous (f)-actin and microtubules throughout three distinct regions of the growth cone: the central (C) domain, the transition (T) zone, and the peripheral (P) domain (Dent et al., 2011). A process of actin polymerization is responsible for growth cone advance, while retrograde actin flow, mediated by myosin II contractility, as well as f-actin buckling, is responsible for filopodial collapse (Lowery & Van Vactor, 2009).

1.4.4 ACTIN

The dynamic properties of actin are responsible for the protrusion, mobility, and exploration of the leading edge of the growth cone. Growth cone filopodia are supported by actin bundles, and lamellipodia are characterized by a branched meshwork of individual actin filaments. A constant process of actin treadmilling is largely responsible for the dynamic behavior in the P domain, where globular (g)-actin is added at the leading, barbed end of f-actin filaments, while disassembly occurs at the caudal, pointed end. F-actin assembly is largely controlled by the ENA/VASP family of proteins which bind to barbed ends in order to antagonize capping proteins as well as to recruit g-actin subunits (Drees & Gertler, 2008). The ADF/cofilin family of proteins is responsible for actin depolymerization, acting at the pointed end to support retrograde flow and
at the barbed end to reveal new, active sites for polymerization (Vitriol & Zheng, 2012). More recently, the pointed-end disassembly behavior of ADF/cofilin has been implicated in the organization of space for the protrusion and bundling of microtubules (Flynn et al., 2012). In lamellipodia, the Arp2/3 complex nucleates actin filaments to control branching, and is also involved in filament assembly (Lowery & Van Vactor, 2009). Pharmacological inhibition of Arp2/3 has shown to disrupt lamellipodial veils and reduce barbed end assembly (Yang et al., 2012). The resulting force of the f-actin assembly pushes the membrane forward while a process of f-actin retrograde flow pulls the actin backwards. Within the T zone, the molecular motor protein myosin II is responsible for regulating retrograde flow. A controlled competition between actin assembly and retrograde flow is largely responsible for determining the extension or retraction of the cytoskeleton. For example, a blockade of myosin II by blebbistatin reduces retrograde flow, therefore speeding up growth cone extension, while an inhibition of ENA/VASP reduces f-actin assembly to produce the opposite effect (Rösner et al., 2007; Drees & Gertler, 2008; Mohamed et al., 2012). Endogenously, the clutch model of growth cone guidance is thought to control the relative contribution of retrograde flow. The model suggests that myosin II engagement at point contacts resulting in decreased rate of retrograde flow, therefore tipping the homeostatic scale towards membrane protrusion due to barbed-end f-actin assembly (Suter & Forscher, 2000, 2001; Lee & Suter, 2008). The clutch model has been demonstrated with catenins, which couple to N-cadherin receptors and directly affect retrograde flow (Bard et al., 2008), as well as the protein shootin1 which regulates retrograde flow by coupling to L1CAM receptors (Shimada et al., 2008).

1.4.5 MICROTUBULES
In addition to actin remodeling, microtubule function is also required for growth cone advance (Tanaka et al., 1995). Microtubules in the P domain act as guidance sensors, while those in the C domain direct growth cone advance (Gordon-Weeks, 2004). As filopodial receptors form adhesions to local cues, exploratory P domain microtubules advance and organize along actin bundles to stabilize the filopodia and interact with the newly formed point contacts (Letourneau, 1983; Lee & Suter, 2008). Dynamic microtubules are involved in growth cone turning, and their blockade has been shown to prevent turning in Xenopus neurons (Buck & Zheng, 2002). Furthermore, microtubule stabilization is necessary for the transport of certain mechanistic guidance signals to the filopodia, and microtubules may act as a scaffold for localized recruitment of signaling molecules during protrusion (Suter et al., 2004). For example, the localized accumulation of Src kinase signaling at adhesion sites is dependent upon the presence of dynamic microtubules and is necessary for growth cone turning in response to a permissive substrate (Suter et al., 2004).

Microtubules are polarized due to their head-to-tail assembly from tubulin heterodimers. Microtubule-associated proteins, or MAPs, regulate polymerization, stability, cross-linking, transport, and other microtubule-related processes. Similar to actin filaments, polymerization occurs at the leading (plus) end, while the tail (minus) end favors depolymerization. Plus-end tracking proteins, or +TIPs, are involved with polymerization. Capping proteins often stabilize the minus end, so microtubule reduction occurs either through rapid phases of depolymerization, also known as catastrophe, or severing into shorter segments which are more prone to degradation (Vitriol & Zheng, 2012). Severing can occur either by local enzymes, such as spastin and katanin, or by physical stress caused by retrograde actin flow, as evidenced in migrating cells.
as well as neuronal lamellipodia (Gupton et al., 2002; Schaefer et al., 2002; Sharp & Ross, 2012).

During the process of engorgement following the initial remodeling of actin in the P domain, microtubules in the C domain migrate into the newly annexed region (Suter & Forscher, 2000; Buck & Zheng, 2002). Invasion of the microtubules is modulated in part by actin, which is necessary to prevent premature engorgement (Burnette et al., 2007). As the growth cone extends, bundling of microtubules in the growth cone ‘neck’ by myosin II, as well as microtubule plus-end assembly, results in consolidation and extension of the axon (Burnette et al., 2008; Toro et al., 2010).

1.4.6 GROWTH CONE SIGNALING CASCADES

The process of signal transduction in the growth cone which interprets extracellular information into cytoskeletal motility involves cascades of kinases, phosphatases, and calcium ions (Hou et al., 2008). Currently, the most studied of these are the Rho GTPases – a class of molecules which control the cytoskeletal dynamics downstream of nearly all guidance receptors (Hall & Lalli, 2010). Proteins in the Rho GTPase family, which includes RhoA, RAC1, and CDC42, serve as molecular switches, cycling between an inactive and active GDP-bound and GTP-bound state, respectively. Upstream signaling to Rho GTPases is accomplished by guanine nucleotide-exchange factors (GEFs) or GTPase-activating proteins (GAPs) (Watabe-Uchida et al., 2006; Koh, 2007). The state of Rho GTPase activity results in either a modulation of the rate of actin polymerization or filopodial collapse via regulation of myosin II activity (Watabe-Uchida et al., 2006). For example, the binding of Ephrin B3 to the Eph4A receptor activates α-chimerin, a
RacGAP, which in turn inhibits growth cone extension, while Rac and CDC42 directly activate the actin nucleator Arp2/3 (Goley & Welch, 2006; Iwasato et al., 2007).

1.5 AXON GUIDANCE THROUGH SUBSTRATE PATTERNING

For over a century, scientists have exploited the cell’s innate ability for topographic and structural guidance through the use of exogenous materials. In 1912, Ross Harrison found that cells placed on a spider web would adapt their shape and movement to the pattern of the individual web fibers (Harrison, 1912). In the past few decades, developments in small-scale fabrication technologies have allowed scientists to create cell culture substrates with deliberate micro- and nanoscale topographic and biochemical features (Voldman et al., 1999; Li & Hoffman-Kim, 2008). *In vitro* studies on surface-modified substrates allow for basic research on the cellular response to different stimuli. Furthermore, these studies provide clues to the mechanisms responsible for cellular behavior *in vivo*, along with optimization guidelines for the development of various medical implant devices. The development of patterned biomaterials is particularly important within the field of neuroregeneration, as fabricated implants may serve to aid in the regrowth of axons following trauma (Norman et al., 2009; Gerberich & Bhatia, 2013; Wrobel & Sundararaghavan, 2014).

Patterned substrates presented to neurons include grooved surfaces, aligned fibers, cell-inspired topographies, pillars and posts, surfaces with varying degrees of roughness, and arrangements of localized biochemical signals (Figure 1). High topographic fidelity is often achieved with laser etching to create grooved surfaces, allowing for neuronal observation in biochemically isotonic, yet structurally diverse environments. Aligned fibers are often used to
mimic development in a biologically-relevant structural environment, such as a nerve or retina (Liu et al., 2010; Kador et al., 2013). Other more biologically-relevant structures can be created by a cell-inspired topography, where surfaces are molded through contact with actual tissue in order to recreate specific cellular shapes. Isotropic materials such as pillars, posts, and substrates with a controlled nanoroughness are often used to study network formation, discern levels of sensitivity to certain features, and identify interactions between biochemical and topographic...
cues (Hoffman-Kim et al., 2010; Kundu et al., 2013). Biochemical localization, whether through the use of printing, microfluidics, or laser patterning, can also be used to control neuronal and growth cone behavior and morphology (Tai & Buettner, 1998; Jang et al., 2010; Khan & Newaz, 2010).

1.5.1 TOPOGRAPHIC GUIDANCE

Grooved surfaces, usually prepared by a process of laser-etching or photolithography, allow for the design of fine-scale, continuous features to study the effects of minor topographical changes on neuronal behavior (Rajnicek et al., 1997). Growth cones are highly sensitive to the seemingly miniscule fluctuations in surface topography. Hippocampal neurons plated on PLL-coated slides with 1 - 4 µm - wide grooves altered their alignment by 90° depending on the groove width: 1 µm and 4 µm grooves caused parallel and perpendicular alignment with the pattern, respectively (Rajnicek et al., 1997). Perpendicular orientation was also cell-specific, occurring in mouse hippocampal but not Xenopus neurons, and was dependent on the function of growth cone calcium channels (Rajnicek & Mccaig, 1997). A response to nanoscale features has also been noted in growth cone substructures and cytoskeleton. Neurons growing on sub-micron, parallel lines will often exhibit aligned filopodia and a polarized compartmentalization of actin in the P domain relative to neurons on a 2D substrate (Johansson et al., 2006; Jang et al., 2010).

The process of electrospinning makes available packed structures of microfibers which can be made from and coated with various biocompatible materials. It has been suggested that a network of aligned fibers can mimic the fibrillar in vivo environment, helping to steer neurons in
a particular direction. As a result, various arrangements of electrospun fibers have been proposed for use in biomedical implants. Aligned bundles of fibers have been tested as nerve conduits, serving to guide DRG neurons through transected peripheral nerve sections (Kim, Haftel, et al., 2008; Biazar et al., 2010). Electrospun fibers can be used to replicate more complex structures as well: RGCs plated on radially aligned fibers were shown to mimic the general structure of the retina, opening a doorway for regeneration following neuronal loss in glaucoma (Kador et al., 2013). Fiber orientation is particularly important in successful guidance; neurites extending from DRG explants aligned themselves with bundles of parallel fibers, and extended significantly farther than those plated on a non-aligned fiber mesh (Kim, Haftel, et al., 2008).

Neurons are also particularly sensitive to substrate width: DRGs plated on fibers with a 5 µm diameter extend significantly farther than on those with a diameter of 30 up to 500 µm (Xuejun & Tresco, 2006).

While it has been repeatedly shown that neurites can be guided by other cell types, such as radial glia, Schwann cells, or adjacent neurons (Marcus & Mason, 1995; Thompson & Buettner, 2006; Malatesta et al., 2008), the relative contributions of surface biochemistry and topography have been difficult to discern. In order to address this issue, researchers have developed techniques which recreate the topography, but not the chemical makeup, of guiding cells. For example, PDMS replicas have been created to resemble the nanoscale topography of Schwann cells (Bruder et al., 2006). DRGs cultured on PDMS preferred Schwann cell topographies over flat surfaces, and neurite growth was aligned with the underlying Schwann cell pattern (Bruder et al., 2007; Richardson et al., 2011). Non-cell-inspired platforms of tissue-based topographic guidance have been developed as well. For example, a polystyrene replica of
the basal lamina, known to play a role in PNS nerve regeneration, (Scheib & Höke, 2013), has proven to be an excellent guide for regenerating neurons (Karlsson et al., 2011).

Microscale and nanoscale pillars, as well as various degrees of nanoroughness, have been presented to neurons as an isotropic topography. Neurons have repeatedly shown an uncanny ability to follow interrupted pillars and posts. When plated on 1 µm tall pillars 1.5 to 4.5 µm apart, hippocampal neurites tend to span the smallest distance between pillars, often in 0° or 90° formations (Dowell-Mesfin et al., 2004). Hippocampal neurites retain the ability to form right-angle networks up to certain pillar dimensions, but as the distance between pillars is increased past 20 µm, the pattern of neurite alignment becomes more stochastic (Hanson et al., 2009). When combined with an overlapping biochemical gradient, the effect of inter-pillar distance on neurite growth informs researchers of whether growth cones respond to biochemical and topographic cues in a competitive or complementary fashion. For example, when hippocampal neurons were plated on an array of pillars, the pillars synergistically enhanced attractive and reduced repulsive guidance by a superimposed gradient of bifunctional netrin-1, while eliminating suppressive guidance by Sema3A (Kundu et al., 2013).

Nanorough surfaces, often characterized by non-aligned features with varying heights in the range of nanometers, have produced mixed results relative to cell adhesion. The seemingly arbitrary nanoscale profile of these substrates is often difficult to reproduce between labs, and the nanoroughness of the in vivo environment is still poorly understood (Kim et al., 2012, 2013; Gerberich & Bhatia, 2013; Park & Im, 2014). One study found that an average feature size of 1 to 5 nm reduced attachment of primary hippocampal neurons on nanotextured titanium nitride
films relative to PDL-coated glass (Cyster et al., 2004). Another study compared primary cortical neuron behavior on surfaces with an average feature size between 10 and 250 nm, and found that the optimal surface roughness for adhesion and longevity exists between 10 and 100 nm (Khan et al., 2005). Nanoroughness gradients have also been implicated in differentiation. PC12 cells, which normally require NGF to form neurite processes and growth cones, were able to differentiate without NGF on glass surfaces modified by chemisorption to create aggregated zones of roughness (Lamour et al., 2009).

1.5.2 BIOCHEMICAL PATTERNING

While topographic surface modification can be used to investigate neuronal behavior by creating biochemically sterile structures, molecular patterning techniques can be used to better understand neuronal responses to various ECM ligands. Many studies have investigated neuronal chemotaxis in homogenous 2D substrates, but molecular patterning in vivo is complex, and the localized segregation of guidance cues is necessary for neurite navigation (Marcus & Mason, 1995; Chung, Shum, et al., 2000; Mortimer et al., 2008; Reese, 2011; Schwarting & Henion, 2011; Molnár et al., 2012). Neuronal migration, differentiation, guidance, and polarization can all be influenced by molecular surface modification in vitro. The surface chemistry of patterned substrates is characterized not only by the type of binding ligand, such as laminin or collagen, as well as the ligand density, but also by the shape and spatial distribution of that ligand (Falconnet et al., 2006). A number of approaches for biochemical patterning have been developed, ranging in price and accessibility, resolution, precision, and scale.
Photolithography, the process of creating a three-dimensional mold through the use of a UV light source, a photomask, and photoresist, is a common approach to surface patterning for biological applications (Whitesides et al., 2001). Usually, polydimethylsiloxane (PDMS) is then cast into the mold to create either a stamp, used for microcontact printing (µCP), or a microfluidic enclosure (Wheeler et al., 1999; Kim, Kwon, et al., 2008). µCP benefits from being approachable and fairly inexpensive, yet is limited in resolution and reproducibility. µCP does allow for higher fidelity features than some other protein deposition methods, such as ink-jet printing (Roth et al., 2004), yet achieving resolutions smaller than 10 µm becomes either prohibitively expensive or requires highly specialized equipment. Once a PDMS stamp is ‘inked’ with protein of choice, the transfer process involves simply pressing the stamp down onto a glass surface. This crude methodology often results in irregular protein transfer, limiting control of precise features and protein density. To address the lack of control over protein density, one group has created stamps with varying densities of sub-micron pillars, although predictably, this method requires more specialized equipment than is available to most labs (Hodgkinson et al., 2012). By relying on liquid, versus dry, protein deposition, microfluidics creates much more reproducible protein-coated surfaces. Microfluidic chambers with specially-designed capillaries allowing for laminar flow have allowed scientists to create diffuse as well as surface-bound molecular gradients in order to study neuronal behavior in response to converging and competing cues (Kim, Kwon, et al., 2008; Park et al., 2013). As with µCP, high resolution features in microfluidics are achievable (Hung et al., 2013), but remain very difficult to produce. Furthermore, due to the intermediate materials involved in photolithographic techniques, the shapes and distributions of features are often limited by the technical constraints of the soft
PDMS, since the stamp or microfluidic chamber has to interact with the glass without buckling (Hui et al., 2002).

Recently, laser-guided surface patterning has allowed for the production of highly precise biochemical patterns. By exploiting the photobleaching properties of certain molecules and cross-linkers when exposed to long-wave light, one group developed a procedure capable of patterning proteins at sub-micron resolutions (Xu & Webb, 1996; Bélisle et al., 2008). The technique, dubbed laser-assisted absorption by photobleaching (LAPAP), has been used to create distinct protein gradients and guide individual neuron attachment (Bélisle et al., 2008; Wissner-Gross et al., 2011). Using a similar technique, another group was able to direct the polarity of hippocampal neurons by altering the size and shape of small, adhesive PDL triangles on the substrate (Scott et al., 2012). The relatively non-invasive nature of laser patterning has even opened the doors for pattern adjustments after cell plating; by guiding fibrinogen absorption using a low-power UV light pulse, a third group was able to change the adhesion zone, and therefore the morphology, of cells in culture (Vignaud et al., 2012). The use of two-photon micro photopatterning (µPP), a technique where a non-adhesive PVA coating is burned away from a glass substrate to allow for localized protein adhesion, has allowed for the creation of narrow channels of fibronectin surrounded by wide, non-adhesive regions of polymer (Doyle, 2009). By isolating individual cells on these confined, 1D lanes, Doyle et al. have shown that narrowly patterned surfaces are superior in recreating 3D, in vivo-like environments than the often preferred, 2D substrates (Doyle et al., 2009). Ultimately, the precise control over feature dimensions and protein composition led us to adapt this photopatterning technique for our own studies.
1.5.3 MECHANISMS OF CELL RESPONSE TO SUBSTRATE PATTERNING

As previously discussed, cells are highly sensitive to miniscule changes in topography, and responses to topography are cell-specific (Rajnicek & Mccaig, 1997). Although the effects of topography or biochemical patterning on neuronal behavior has been investigated by many labs, few studies have examined the mechanisms responsible for these effects. Therefore, while multiple ideas have been proposed to explain the various morphological and behavior changes witnessed in neurons on patterned substrates, the individual contributions of these mechanisms remain unclear.

One possible mechanism for topographic guidance proposes that changes in the 3D surface profile alter the conformation with which proteins interact with the substrate (Khan & Newaz, 2010). This change in protein conformation, in turn, would affect the way in which the biochemical environment is presented to extending neurons. Changes in protein orientation are particularly likely to play a part in responses to nanoscale topography, where feature size is similar in magnitude to the size of various proteins. For example, single molecules of aggrecan, a type of CSPG, and laminin have lengths of about 350 and 80 nm, respectively (Engel et al., 1981; Harder et al., 2010). If patterned on a nanorough surface with an average feature size of 200 nm, one can reasonably expect that the molecular conformation would be different than that on a 2D surface. Similar to the lock and key ligand-receptor model, proteins absorb more readily to a surface patterned with the imprint of either certain parts or the entire structure of that protein (Shi et al., 1999). In fact, nanometer-scale topographies have been found to affect the biological function, motility, and morphology of a protein coating (Lim et al., 2005; Song & Chen, 2007).
Patterned substrates may also act directly on the cytoskeleton by selectively exerting and relieving pressure in various regions. Crudely, these cytoskeletal rearrangements may result in, for example, highly aligned cells. It has been proposed that a minimization of cytoskeletal distortion would be energetically favorable, and therefore, taking the shape of a guiding structure would result in the least amount of stress on stiff components such as microtubules (Dunn, 1991). Parallel grooves, as well as biochemically patterned lanes, are likely to force a cytoskeletal rearrangement in neurons and result in alignment along the pattern (Rajnicek et al., 1997; Miller et al., 2002; Jang et al., 2010; Liu et al., 2013; Su et al., 2013). As previously discussed, hippocampal neurons have shown to prefer perpendicular alignment in certain situations, but it has recently been suggested that stress in the filopodial cytoskeleton prevents perpendicular guidance as a function of increased groove depth (Rajnicek & Mccaig, 1997; Chua et al., 2014). Surface adhesions in filopodia, in particular, are known to create patterns of mechanical stress, and the act of cell spreading can induce a rearrangement of the cytoskeleton through intracellular forces (Walboomers et al., 1998; Janmey & Weitz, 2004; Koch et al., 2012). Cells can also change their gene expression patterns following an initial cytoskeletal reorganization (Itano et al., 2003). For example, culturing cells on single ECM islands of varying sizes could force them into growth, apoptosis, or selective differentiation (Dike et al., 1999; Eyckmans et al., 2012). In the case of neurons, cytoskeletal tension is important for growth cone guidance and is partly responsible for axon elongation (Loverde et al., 2011; Suter & Miller, 2011; Rauch et al., 2013).

A forced reorganization of the cytoskeleton would go hand-in-hand with changes in receptor localization, as well as their downstream signaling cascades. On confining patterns, cells
and growth cones would become highly polarized and exhibit different functional processes compared to their non-patterned counterparts. The most obvious example is the rearrangement of adhesion sites; changing the dimensions of a substrate alters the adhesion patterns on a cell membrane, reflecting reorganization in both the cytoskeleton as well as cell surface receptors (Chen et al., 2003; Doyle et al., 2009). In addition, forces applied to adhesion sites have the ability to directly strengthen the biochemical scaffold involved in the adhesion. “Catch” bonds between integrins and the ECM become strengthened by lateral forces exerted by the cytoskeleton, and an increase in FAK phosphorylation, as well as Rac activation, were seen as a response to tension (Katsumi et al., 2002; Chen et al., 2004; Moore et al., 2010).

1.5.4 CELL MODELS

When choosing a cell model to study neuronal outgrowth, researchers must often weigh the relative importance of many factors, including biological relevance, culture heterogeneity, and cell size. For example, large Aplysia growth cones are often used in studies identifying cytoskeletal components of the growth cone, but the model holds little relevance for regeneration since the glial scar is specific to warm-blooded animals (Burnette et al., 2007; Lee & Suter, 2008). DRGs, on the other hand, are often incorporated in regenerative outgrowth studies. While not a true CNS neuron, DRGs are sensitive to the glial scar, have shown regenerative potential in the spinal cord, and have been used to study growth on patterned substrates (Bruder et al., 2007; Hodgkinson et al., 2012; Riblett et al., 2012; Joddar et al., 2013). DRG growth cones are also relatively large, allowing researchers to easily identify the details of their shape (Koch et al., 2012). The downfall of DRGs is their relatively heterogeneous culture...
(Neacsu & Flonta, 2006), as isolating growth cones becomes more difficult on patterned substrates. Unlike DRGs, CGNs offer a fairly homogeneous culture, lacking the discrepancies between various neuronal and glial subtypes (Bilimoria & Bonni, 2008). The relatively simple architecture of the cerebellar cortex provides CGNs with a highly stereotypical pattern of organization and readily distinguishable axons and dendrites (Bilimoria & Bonni, 2008). Finally, CGNs are considered an easily transfectable cell, which helps for visualizing certain cytoskeletal and molecular processes. Conversely, the relatively small size of CGNs and their growth cones may inhibit certain spatially sensitive studies, although these limitations can be overcome with high magnification and precision optics.

1.6 AIM OF THE STUDY

By breaking down growth and development into single-cell responses to guidance cues, we can explore the mechanisms involved in neuronal behaviors seen in vivo. With the use of precise substrate patterning, we can better model complex in vivo environments, such as the optic chiasm or the glial scar. It has been established that growth cones can alter their size as well as rate of movement as a response to pattern dimensions. Therefore, in this study, we aimed to (1) establish a model system where we could control neuronal growth cone morphology in a reproducible way, (2) test whether altering growth cone morphology would lead to changes in growth cone velocity, and (3) create an assay which would allow us to test whether growth cone morphology affects sensitivity to inhibitory chemical cues. In addition to exploring the mechanisms of neuronal guidance, we propose that our model serves as a
platform to develop and optimize structural cues to help engineer neuronal regeneration following CNS injury.

2 PATTERN DEVELOPMENT

2.1 INTRODUCTION

The process of determining the influence of growth cone morphology on behavior required an ability to manipulate growth cone shape while evaluating outgrowth in real time. In the past, changes in growth cone morphology have been observed following pharmacological and genetic manipulation of cytoskeletal proteins (Lee & Suter, 2008; Geraldo & Gordon-Weeks, 2009; Kurklinsky et al., 2011), as well as exposure to substrate patterning (Clark et al., 1993; Tai & Buettner, 1998; Messa et al., 2009; Féréol et al., 2011). In order to isolate the effects of growth cone morphology as the primary causal mechanism in our study, we chose to focus on substrate geometry due to its relative non-invasiveness as compared to direct interference with intracellular mechanisms. Other methods, such as pharmacological intervention, may work to form phenotypically small versus large growth cones, but the resulting changes in behavior are difficult to unambiguously attribute to growth cone morphology versus the pharmacological intervention itself.

In order to test our hypothesis that decreased substrate geometry could reduce growth cone size, growth cone adhesions had to be entirely confined to the spaces defined by our patterns. Furthermore, precise control of pattern features was required for confinement of growth cones into regions smaller than their native size on an unpatterned substrate. Therefore,
our assay required (a) the ability to create features with a sub-micron level of accuracy, (b) a consistent concentration of permissive protein to guide growth cone advance, (c) a strict boundary to block any adhesion outside of the patterned region, and (d) isolated, easily visualized growth cones. To test our next hypothesis of whether changes in growth cone shape affect growth rates, our assay would also need to (e) allow for live imaging of multiple time points. After some initial trials with various methods, we found the previously discussed µPP technique to be well-suited to our needs. Initially developed by Doyle et al. to study one-dimensional migration in fibroblasts, the technique relies on a two-photon microscope to selectively expose regions of glass, removing a non-permissive layer of PVA (Doyle, 2009; Doyle et al., 2009). The exposed areas of glass can then be coated with various ECM proteins, forming adhesive regions to allow for cell attachment. By utilizing glass-bottom dishes, cell growth in the patterns can be observed in real time using phase contrast microscopy.

2.2 PATTERN FABRICATION

A protocol for pattern fabrication was largely adapted from one published by Andrew Doyle (Doyle, 2009), although differences in hardware, software, targeted cell type, and types of patterns resulted in various important changes. Patterns were created on individual MatTek© 35mm dishes with a 14mm glass bottom (P35G-1.5-14-C) due to their compatibility with the spincoating process and required microscope objectives, as well as their support of multiple-day cell culture. Before photoablation, dishes needed to be silanized and coated with PVA. After photoablation, dishes were quenched to prevent autofluorescence, coated with laminin, and seeded with cells (Figure 2, Figure 3).
2.2.1  PREPARATION OF GLASS SURFACES

Figure 2. Surface preparation and PVA coating of substrates. Lines connect glass surface chemistry (right) with the corresponding coating step (left). Molecular structure of PVA adapted from Doyle et al. (2009).
First, the glass surfaces were cleaned by acid washing. Under a fume hood, 250 µl of 50% solution of HNO₃ was added to each dish using a Pasteur pipette. The acid was spread to make sure the entire glass area was covered, and the dishes incubated for 25 min at room temperature (RT). Next, dishes were placed in a large glass container and exposed to a steady flow of deionized (DI) water for a minimum of two hours. The acid-washing step helps lift various contaminants from the glass surface, while rinsing with DI water insures the complete removal of these soluble contaminants and exposure of a uniform glass surface.

H⁺ residues were replaced with OH⁻ in order to improve silane adhesion to the glass surface. After the water was aspirated, dishes were incubated with 250 µl of 200 mM NaOH for 15 min at RT. The NaOH was then aspirated and dishes were rinsed twice with 2ml DI water.

Since PVA is not inherently adhesive to a glass surface, a silane coating was necessary. We used triethoxysilylbutraldehyde (TESBA), an aldehyde-terminated silane which could adequately form a bond between the OH⁻ groups on the glass surface and the PVA. Immediately before use, a 1% w/v solution of TESBA is prepared in Ethanol using a glass pipette and incubated for 5 min. Next, dishes were incubated with 1% TESBA for 5 min, after which point the solution was aspirated and dishes were washed twice with ethanol and once with DI water. Silanized dishes were then cured at 65°C for a minimum of two hours, and then stored in a desiccated container for up to 1 month at 4°C.
An even coating of PVA was achieved through the use of spincoating – a process which uses centrifugal force to evenly spread thin layers of various mixtures on a substrate.

Since PVA dissolves poorly in water, it was necessary to heat the PVA and water mixture to achieve a reproducible concentration of the polymer. 2.835 g of 98% hydrolyzed PVA was added to a 50ml conical tube, and DI water was added to bring the final weight to 50g. A stir bar was placed inside the conical tube, and the tube placed inside a glass beaker filled with boiling water. After continuous stirring and applied heat, the dissolved PVA mixture was passed through a 0.2 µm filter using a vacuum. Filtration was necessary to remove any undissolved PVA pellets which could later form significant artifacts during the spincoating process. This PVA solution could be stored for at least 6 months when sealed at RT.

Once cooled, 4.5 ml of the dissolved PVA solution was combined with 600 µl of 2N HCl and mixed by vortexing. The bubbles formed by vortexing the viscous mixture were removed by exposure to a vacuum chamber for 1 hour, and then by centrifugation for 3 min at 2000 G. Dishes were then incubated with 300 µl of the mixture, and spun at 500 rpm for 5 sec and 5000 rpm for 60 sec. Spinning for less time or at lower speeds resulted in PVA which was noticeably uneven, resulting in visible grooves, while faster spinning or longer spin times resulted in a layer where the PVA was too thin, making the final pattern very difficult to discern under a wide-field microscope.

After spincoating, dishes were stored for a maximum of 2 weeks at 4°C before photoablation. Crystallization or loss of surface hydrophilicity was prevented by placing an open container of 5M NaCl in the storage container in order to properly regulate humidity. NaCl
solutions maintain a humidity between 50 and 60 %, which is optimal for reducing PVA crystallization and loss of surface hydrophilicity (Doyle, 2009).

2.2.3 PHOTOABLATION

Figure 3. Generation of laminin-coated patterns using laser photoablation. (A) A thin layer of PVA on top of glass is selectively ablated using repetitive high-power laser scanning in a Region of interest. Laminin selectively adheres to glass exposed by the ablation process. (B) AlexaFluor-488 laminin (purple) after adhesion to photoablated dish. Bottom: moving average (bin of 5 pixels) of a fluorescence intensity plot along the white dashed line divided by overall average intensity. Scale bar = 10 µm. Adapted from Smirnov et al. (2014).
Achieving proper photoablation required a precisely calibrated multiphoton beam to target the PVA layer without affecting the glass below (Figure 3A). A Leica SP5 Multi-Photon (dual beam with OPO)/Confocal Microscope was used along with standard Leica operating software. Dishes were placed on the stage and a focal plane located using reflectance with a low power 633 nm laser and 40x oil immersion objective. In order to account for z-drift, a reflectance-based autofocus routine was set up to scan 100 fields in a 10 µm Z-stack at 1600 Hz and a 16x16 resolution. Optimal ablation was found to occur with an offset of -1 µm from the focal plane. Regions of interest (ROIs) were drawn to indicate the intended regions for ablation, and an 805 nm, two-photon laser scanning at a 1024x1024 resolution ablated PVA regions. The baseline scan frequency, power, and gain for the laser were set to 200 Hz, 50%, and 50. Ablation of one ROI (Figure 4A) often needed to be repeated in different Z planes to accommodate for slight tilt in the glass-bottom dish. The ablation intensity was determined by imaging the pattern using low-power 633 nm reflectance light simultaneously with multiphoton scanning. Low ablation power would result in visibly incomplete ablation (Figure 4A), while high ablation power would lead to scorching of the glass as well as ablation outside of the designated region of ROI (Figure 4C). Ablation power was positively correlated with laser power and gain and negatively correlated with scanning frequency. Proper ablation was highly sensitive to the accuracy of these values, allowing for a parameter window of about 5%, and calibration was often necessary to account for fluctuations in laser alignment, base power output, and various environmental inconsistencies.

The photoablation process was automated using the Leica MatrixScreener software package. After the optimal settings for ablation were determined, an array of tiles with a 3%
overlap was defined to reproduce the ablation process in a 2 by 1 mm region. After an initial autofocus scan of the matrix’s corners, each individual tile underwent an autofocus routine and followed by an ablation routine.

Dishes were quenched in order to prevent autofluorescence of the PVA during imaging, reduce any reacted aldehydes to attain a stronger bond between PVA and the glass surface, and to block any free radicals produced during photoablation. Within two hours following photoablation, 2 ml of 200 mM Ethanolamine buffer (200 mM EtOHNH₃, 100 mM NaH₂PO₄, pH 8.0) was added to each dish. 20 µl of an NaBH₄ solution, prepared by mixing 40 mg of NaBH₄ in 1 ml of 1M NaOH, was then added to the ethanolamine solution and incubated in dishes for 8 min. Dishes were then washed three times with water and stored for up to one month at 4°C in 0.04% Sodium Azide in PBS to prevent bacterial or fungal growth.

We used contact AFM quantification in order to characterize the sub-micron topography of the ablated patterns. Briefly, a contact AFM reads surface topography by detecting changes in reflected signal from a small cantilever which makes direct contact with the substrate during a back-and-fourth horizontal scan. We determined that the PVA layer was approximately 100 nm thick, resulting in a 100 nm wall between the ablated region and PVA (Figure 5A). A closer examination of the ablated layer revealed repeating parallel lines less than 100 nm in width aligned with the direction of laser scanning during ablation (Figure 5B).
Figure 4. Photoablation of parallel channels with varying widths. Left: 633 nm reflected image. Right: brightfield image. A: pattern with insufficient ablation, with characteristic punctate patterns in ROI regions (outlined purple and green). B: Ideal level of ablation. Boundaries between ablated and non-ablated regions are clear, and surface inside lanes is uniform. C: Overly intense level of ablation. Ablation boundaries extend past channel ROIs, and light scattering implies scorching and roughening of the glass surface. Scale bar = 10 µm.
Figure 5. Topographic image of pattern using AFM. Height in an AFM image of a pattern represented by relative intensity. Top: the PVA layer is approximately 100 nm high. Bottom: zoomed in scan of area designated by violet square in top image. Parallel lines in pattern represent artifacts, approximately 20 nm in height, likely created by etching of glass surface. Modified from Smirnov et al. (2014).
2.2.4 PROTEIN ADHESION AND CELL CULTURE

Laminin, a well-known permissive ECM ligand (Buettner & Pittman, 1991), was chosen as the protein coating to guide neurite adhesion and growth. Laminin was first conjugated with Alexa Fluor-488 dye according to the supplied protocol, then diluted to 25 µg/ml in PBS with 0.1% Pluronic F-127 added in order to prevent nonspecific binding to the unablated regions of the substrate. Dishes were incubated for 30 min at RT and washed three times with PBS. Fluorescent imaging confirmed that laminin was bound exclusively to the ablated region, and the fluorescence intensity of the signal had remained consistent throughout the pattern, indicating that laminin had settled evenly within the ablated surface (Figure 3B).

CGNs were chosen as a cell model for neuronal growth cones. CGNs were prepared from postnatal 5-day-old C57BL/6 mice using standard procedures (Wang et al., 2008). Dissociated cells were plated at low density (30,000 per well) on laminin-coated patterns and 2D unpatterned laminin-coated glass dishes in Neurobasal-A medium containing B27 (1:50, v/v) supplement, glutaMAX (1:100, v/v, Life Technologies), 1X Pen/Strep, and 25 mM KCl. 24h after plating, CGN cell bodies had adhered to the laminin-coated lanes, and were able to send out neurites which followed the lanes as well. Furthermore, neurites stayed within their own lanes and did not form adhesions with the PVA (Figure 6). In 2D, CGN growth cones varied in width between around 3 and 10 µm, making them a suitable candidate for our given pattern dimensions. Even after neurite sprouting, CGN cell bodies often migrated along patterned lanes as well as on 2D dishes.

2.2.5 DISCUSSION
By employing the technique of µPP, we were able to create adhesive channels with a submicron resolution surrounded by a non-adhesive PVA surface. The fabrication of these patterns in glass-bottom dishes allowed us to perform high-resolution, timelapse imaging of CGN growth cones extending inside the patterned lanes. Our experiments greatly benefitted from incorporating µPP instead of other more common patterning techniques. µCP, for example, while relatively simple, does not allow for easy fabrication of sub-micron features. Both µCP and microfluidic patterning techniques rely on a mask which serves as a light barrier for the fabrication of 3D features in a photoresist substrate. Many services exist which can fabricate these masks based on user-defined blueprints, but the lowest resolution at which features are usually guaranteed before climbing drastically in price is 10 μm (CAD/Art Services, Inc). These masks are often fabricated by printing ink on film, while the creation of higher-resolution masks requires a more complex fabrication process, such as electron beam etching or selective deposition of various metals on a glass or quartz surface (Charest et al., 2006; Falconnet et al., 2006). Due to the complexity of the fabrication process, high resolution masks are often either prohibitively expensive or require highly specialized equipment. Recently, one group found an inexpensive workaround to make sub-micron lanes by using fairly cheap diffraction gratings as a master to create PDMS stamps, but this method lacks the flexibility in pattern design required for our experiments (Hart et al., 2013). Design flexibility is a general problem of PDMS and microfluidic chambers as well. The design of a PDMS structure is constrained by a number of physical properties due to the elasticity of the material and its behavior during contact with the substrate. Unwanted contact and poor patterning can occur due to various stamp deformations, including roof collapse, buckling, lateral collapse, pull-off artifact and surface tension (Hui et al.,
2002). µPP, on the other hand, lacks these material constraints and is therefore gives the user much more freedom in designing specific features.

Photoablated patterns are individually time-consuming, but allow for easy tuning of feature geometries between experiments. A lithographic photomask can be reused to make multiple PDMS stamps or microfluidic chambers, but any alterations to patterned features require the fabrication or purchase of a new mask. Conversely, laser patterning is more time-consuming, requiring each dish to undergo a photoablation process, yet the patterns are easily changed by altering the ROI design, making the initial troubleshooting process much less expensive. µCP also lacks the necessary control over regional protein concentration, and lanes often have too many artifacts for proper quantification of individual growth cones, while we found the laminin concentration in our patterns to be both reproducible and consistent.

µPP achieves proper growth cone confinement by creating adhesive lanes surrounded by a non-adhesive substrate. µCP, on the other hand, requires a relatively adhesive surface to enable protein attachment, often resulting in nonspecific cell adhesion. Poly-L-Lysine (PLL), for example, is often used as a base coating prior to µCP. While certain cells may prefer ECM proteins to PLL alone, PLL is well-documented in its ability to act alone for neuronal attachment and growth (Kelly et al., 2010). Therefore, neurons on surfaces patterned by µCP may sometimes exit their guided channels, and true growth cone confinement is not achievable due to adhesion formation outside of a patterned region.

Initially, patterns were designed with interspersed circular nodes to provide large adhesive regions for cell bodies (Figure 3B). During development, CGN neurons are highly motile,
and their process of migration has been described in detail (Komuro & Rakic, 1995). Both in vivo and in vitro data has shown CGN migration along glial fibers, and migration is not limited to cells which have not yet differentiated or sprouted any neuronal processes (Gregory et al., 1988). Therefore, similar to fibroblasts on confined channels which move in a manner imitating 3D migration along fibers (Doyle et al., 2009), CGN cell bodies often left the patterned circles and migrated along the confined space.

While the photoablation process served to create a biochemical pattern of laminin, it is important to note that neurons were also exposed to minor changes in topography. A 100 nm edge of PVA gave the patterns a distinct ‘valley’ shape, with neurons adhering strictly inside a groove (Figure 4A, Figure 6A). While groove geometry has shown to play a role in neuronal outgrowth and alignment (Rajnicek & Mccaig, 1997; Rajnicek et al., 1997), groove height due to photoablation is at least an order of magnitude smaller than that tested previously. Furthermore, previous studies using grooved topography lacked any biochemical boundaries or distinct non-permissive regions, both of which are present in our patterns. Finally, photoablated lanes were separated by a distance of at least 20 µm, making it nearly impossible for growth cones to travel in a perpendicular manner.

An unavoidable consequence of ablation was the appearance of tiny parallel lines representing a ‘footprint’ of the scanning laser (Figure 4B). These lines were likely either remnants of unablated PVA and/or small etchings into the glass coverslip. Unfortunately, without AFM quantification on each pattern, the existence of these features was unpredictable, and it is therefore possible that they somehow affected neuronal growth. Nanotopography on a similar
scale, 50-250 nm, has shown to alter protein adsorption and alignment, as well as the propensity for healthy cell growth (Khan et al., 2005; Song & Chen, 2007; Park & Im, 2014). Therefore, while the effect was likely minimal, it is important to keep in mind the possible influence of substrate nanoarchitecture on growth and guidance. Unfortunately, these nanoscopic variations are not unique to µPP, as most patterning methods are expected to have minor artifacts which are often ignored, especially when they are more than an order of magnitude smaller than the features being tested.

3 GROWTH CONE RESPONSE TO CONFINEMENT

3.1 INTRODUCTION

While much correlative evidence has been found linking growth cone morphology and behavior (Mason & Erskine, 2000), no causal relationships between the two have been established. Small, streamlined growth cones exhibited by RGCs in the optic nerve and tract have higher overall velocities and spend less time pausing, while growth cones in the optic chiasm are larger and take a longer time to advance (Bovolenta & Mason, 1987; Mason & Wang, 1997). It could then be true that smaller growth cones are, in general, more prone to quicker extension, while larger ones extend more slowly. Another possibility is that the extracellular environment induces changes in both morphology and behavior, but the changes are not directly dependent on each other. Finally, the adaptation of a large, complex morphology could be a mechanism which growth cones use to interpret an elevated number of ECM cues in order to speed up their

1 Note: Significant portions of this chapter are adapted from Smirnov et al. (2014) with permission from Elsevier.
travel time. This last scenario would predict that smaller growth cones may move even more slowly through the optic chiasm than large ones, while larger growth cones would advance more quickly through the optic nerve and tract.

Establishing a causal relationship between growth cone morphology and behavior is difficult in vivo. Pharmacologically induced changes in morphology would likely alter multiple signaling cascades, making any results too ambiguous. By altering growth cone morphology using in vitro patterning, we can isolate various observable morphological changes due to dimensional constraints exerted by the substrata. Therefore, by studying growth cone behavior in neurons plated on patterns fabricated using µPP, we can discern the direct impact of morphology on growth cone behavior. A large portion of this study has been published (Smirnov et al., 2014).

3.2 METHODS

GLASS-BOTTOM DISH PREPARATION. Dishes were prepared according to procedures outlined in section 2.2.1. Briefly, glass-bottom (MatTek) dishes were washed with 50% HNO3, treated with 200 mM NaOH, rinsed with H2O, and silanized using 1% triethoxysilylbutraldehyde (Gelest) in ethanol. Surfaces were washed with ethanol and H2O and allowed to cure for 3 hours at 65°C. A 5.6% PVA solution was prepared by dissolving PVA (molecular weight ≈ 98,000; 98% hydrolyzed; Sigma-Aldrich) in H2O at 90°C and filtered through a 0.2 µm filter. 2N HCl was added to the filtered PVA at a 1:8 ratio. The mixture was spin-coated onto the silanized glass-bottom dishes at 7000RPM for 45s and the dishes were stored at 4°C for up to one month.
PHOTOABLATION. A Leica SP5 Multi-Photon (dual beam with OPO)/Confocal Microscope was used for photoablation. ROIs were generated using Leica operating software. A 63x NA 1.4 oil immersion objective was used during ablation. Digital zoom was set to 4× in order to decrease amount of z-tilt in the field of view. Autofocus was performed using reflectance from a low power 633nm laser. Ablation was achieved via a two-photon laser at 800nm and ≈ 35% power, scanning at 100Hz at a 512x512 resolution. These settings allowed us to burn away PVA but prevent scorching of the glass surface. Leica MatrixScreener software was used to automate the process in order to create consistent, tiled patterns. To reduce autofluorescence, dishes were quenched for 8 minutes with 2 ml of 0.5% NaBH4 in 200 mM Ethanolamine buffer (200 mM EtOHNH3, 100 mM NaH2PO4, pH 8.0) and then washed 3x with PBS.

LAMININ LABELING AND ATTACHMENT. Laminin was conjugated with Alexa Fluor-488 dye (Life Technologies) using supplied protocols. Briefly, 50 µl of 1M Sodium Bicarbonate was mixed with 500 µl of 1mg/ml laminin, and the solution then added to a vial of reactive dye including the Alexa Fluor 488 carboxylic acid, succinimidyl ester, and dilithium salt. After 1 hour of reaction time, unconjugated dye was removed using purification resin in spin columns, and the final degree of labeling was measured using absorbance at 280 and 494 nm. Fluorescently labeled laminin was diluted to 25 µg/ml in PBS with 0.1% Pluronic F-127 (Life Technologies) and added to coverslips for 30min at room temp. Dishes were washed 3x with PBS and kept in PBS until use.

ATOMIC FORCE MICROSCOPY (AFM) IMAGING. Topographic Images of the ablated regions were acquired with a Solver Next (NT-MDT) scanning probe microscope operating in oscillating mode.
CELL CULTURE. Cultures of dissociated mouse CGNs were prepared from postnatal 5-day-old C57BL/6 mice as described previously (Wang et al., 2008). Dissociated cells plated on laminin/coated patterns and 2D unpatterned laminin-coated glass dishes in Neurobasal-A medium containing B27 (1 : 50, v/v) supplement, glutaMAX (1 : 100, v/v, Life Technologies), Pen/Strep, and 25 mM KCl.

SEM PREPARATION AND IMAGING. Coverslip cultures were fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 0.12 M sodium cacodylate buffer, pH 7.3, postfixed with 1% OsO4 in the same buffer, dehydrated in an ethanol series dried using a Samdri-795 CO2 critical point dryer (Tousimis Research Corp, Rockville MD). The dried coverslip cultures were coated with 5nm of gold in an EMS 575-X sputter coater (Electron Microscopy Sciences, Hatfield PA) and imaged with a Hitachi S-3400 N1 scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton CA).

TIME-LAPSE MICROSCOPY. Phase contrast images were obtained using a Nikon Eclipse TE2000-E inverted microscope equipped with a 37°C and 5%CO2 incubation chamber, 63x oil objective, and a motorized stage (Prior Scientific). Metamorph software (Molecular Devices) with the multidimensional acquisition plugin was used to capture images and control all hardware. Images were acquired using a 63x oil objective and digital camera (Hamamatsu Photonics). Regular time-lapse imaging was performed in ~20 locations at 5min intervals over two 24-hour periods, while high time-resolution imaging was performed in one location at 5s intervals. Laminin fluorescence was detected using epifluorescence illumination with a mercury lamp and a 488nm filter cube.
**IMAGE PROCESSING AND ANALYSIS.** Image processing was performed using ImageJ (Schneider et al., 2012). Image stacks were imported and drift-stabilized using the Template Matching plugin. The MtrackJ plugin was used to track growth cone position, while area and aspect ratio (calculated as a ratio between major and minor axes) were measured by manually tracing each growth cone in each frame. Data analysis was completed using Matlab 2013a (MathWorks).

103 growth cones in were measured on uniform channels and in 2D between 24 and 72h after plating. Data was accumulated from nine individual experiments (dishes), although each experiment did not contribute an equal amount of growth cones per group. The wrist of the growth cone was used as the coordinate for growth cone position. Directionality was determined by setting a reference point at the very beginning of each the channel. For unpatterned 2D controls, the reference point was the first position in frame. Movement towards and away from the reference resulted in a negative and positive velocity, respectively. Error due to turning was negligible in the channels since there was no sideways movement without significant forward or backward displacement. Significant turning was noted and manually corrected for in control growth cones. Average velocity for each axon was calculated as a total displacement between the first and last recorded position divided by elapsed time, and in-group velocities were analyzed for average and standard error. Growth cones in 2D tended to grow in straight lines, but significant turns in growth trajectory were incorporated into displacement measurements.

Area and aspect ratio were measured in each frame and then averaged for each individual growth cone. These values were then averaged between all growth cones in each
group to avoid bias due to varying sample size. Cumulative probability histograms were plotted using raw instantaneous area measurements.

Instantaneous velocities at each timepoint were calculated as a change in position along the axis of the channel from the previous frame to the following frame divided by time. Growth cones moving at a rate higher than 0.2 µm/min for at least three consecutive frames (15 min) were considered advancing, while velocities above 0.2 µm/min for fewer than three frames and velocities between 0.2 µm/min and -0.2 µm/min were considered paused. Growth cones with velocities less than -0.2 µm/min were considered to be retracting.

33 growth cones were measured on patterns of channels interspersed with circular nodes. Instantaneous velocity was calculated as a displacement along the axis of the channel of growth cones in each consecutive frame divided by time between frames. Directionality was again determined using a reference point. Growth cone position reflects distance to the closest node center. Average velocities and standard errors were calculated for each position. A moving average function (bin=4) was used to smooth velocity data before plotting. Growth cone areas were also grouped in 1 µm bins and averaged for each position.

**STATISTICS.** No significant differences in results were seen in growth cones imaged at 24-48h and 48-72h, resulting in data from both days being grouped together. Statistical analysis was performed in MATLAB 2013a (Mathworks) with a multiple comparison test using a one-way ANOVA. Results were considered statistically significant if p < 0.05.

3.3 RESULTS
To examine growth cone responses to precisely defined submicron-scale features, we used µPP (Figure 3A) to fabricate laminin-coated channels surrounded by non-adhesive regions of PVA. The scale of pattern features was chosen to best approximate the range of CGN growth cone sizes in vitro, and neurons were plated on patterns consisting of parallel lanes of varying widths, as well as narrow lanes interspersed with rounded nodes.

![Figure 6](image-url)

**Figure 6.** Pattern design using parallel channels of varying widths. A: Schematic representing neurons extending on pattern. B: Brightfield images of CGN growth cones confined to laminin-coated, ablated channels.

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### 3.3.1 INCREASED CONFINEMENT ALTERS GROWTH CONE MORPHOLOGY
Figure 7. Channel width controls growth cone morphology. (A) Left to right: representative brightfield images of growth cones in 1.5 µm, 6 µm, and 12 µm channels. Red dashed outline indicates typical area measurement. (B) Representative SEM images of growth cones in different channels. Top to bottom: 1.5 µm, 6 µm, and 12 µm channels. (C) Average growth cone area and SEM in each group. 2D indicates unpatterned substrate. F=12.9. (D) Average growth cone aspect ratio (major/minor axis) and SEM in each group. 2D indicates unpatterned substrate. F=21. N = 19, 15, 22, 19, and 28 for 1.5 µm, 3 µm, 6 mm, 12 µm, and 2D, respectively. (E) Cumulative distribution histogram of individual growth cone area measurements. *p < 0.05 when compared to 12 µm and 2D groups. #p < 0.05 when compared to 2D group. Adapted from Smirnov et al. (2014).
In order to evaluate the effect of varying degrees of confinement on growth cone size, we created patterns consisting of parallel channels 1.5 µm, 3 µm, 6 µm, and 12 µm wide (Figure 6A). Postnatal day 5 CGNs were plated and allowed to settle for 24h to ensure proper adhesion to the patterned substrate. Cells were then imaged using time-lapse phase contrast microscopy for 48h (Figure 6B). CGNs consistently extended their neurites along the laminin-coated patterns, and neurites were never observed entering unablated regions. While growth cone filopodia and lamellipodia often explored immediately outside and above the confined pattern,
growth cones themselves were confined to their respective channels, appearing larger in wider channels (Figure 7A).

SEM examination revealed that channel width also affected the three-dimensional shape of the growth cones: narrower channels tended to produce growth cones with a thick, tube-like appearance and often vertically-oriented filopodia, while wider channels tended to produce flatter growth cones with more lamellipodia (Figure 7B). Imaging of fixed growth cones in
confined channels also indicated an enlarged C-domain (Figure 9), but we chose to focus on the growth cone’s area, as opposed to volume, due to its accessibility of observation in timelapse, brightfield imaging.

The area of each growth cone in each frame of the time lapse video was measured by tracing the outline of the growth cone (Figure 7A). We found a significant effect of confinement on growth cone area: growth cones were largest on 12 µm channels and unpatterned, laminin-coated glass, and significantly smaller on narrower channels. However, we found no significant difference in area between 1.5 µm, 3 µm, and 6 µm channels (Figure 7C).

We then plotted the cumulative distributions of individual area measurements of all growth cones at all times for each lane width (Figure 7E). Growth cone area in all channels had a wide distribution, ranging from 2 - 130 µm², with most falling between 5 - 75 µm². All but the 12 µm channels, including the unpatterned 2D group, have a large portion of small growth cone areas. In contrast, there was an increased proportion of larger growth cone areas recorded in 12 µm channels, although these values were recorded from a few growth cones and therefore are not significantly reflected in the averages in Figure 7C. Although area was widely distributed, the distributions in each growth cone were relatively similar (Figure 8).

Growth cone aspect ratio was computed using the major and minor axis calculated from each trace. Average aspect ratio was significantly increased in 1.5 - 6 µm channels compared to 12 µm and unpatterned surfaces, as confinement results in a narrowing and elongation of growth cones (Figure 7D).
Figure 10. Growth cone velocity is not affected by global variations in channel width. (A) Representative brightfield image indicating wrist (white arrow) of growth cone used as marker for position. (B,C) Distribution and average of growth cone velocities based on total displacement in each group. N=19, 15, 22, 19, and 28 for 1.5µm, 3µm, 6µm, 12µm, and 2D, respectively. (D) Smoothed histogram of instantaneous velocities from all growth cone positions. Adapted from Smirnov et al. (2014).

3.3.2 GROWTH CONE VELOCITY IN UNIFORM CHANNELS IS INDEPENDENT OF WIDTH
To examine how confinement influences extension rate, we compared growth cone velocities in each channel. For a consistent measure of location, growth cone position was measured at the wrist (Figure 10A), the least dynamic point in the structure. The average velocity for each growth cone was calculated as the total displacement between the positions observed in the first and last frame in which a given growth cone was visible divided by the elapsed time. Growth cone velocity ranged from 0.08 to 1.6 µm/min, and velocities were relatively tightly grouped in narrow channels, but had a wider distribution in the 12 µm channel (Figure 10B). When calculated from average displacement between consecutive frames instead of total displacement, velocities had a wider distribution in the 2D condition (Figure 10D). However, we found that average velocity was independent of channel width (Figure 10C). Furthermore, we
Figure 12. Instantaneous growth cone areas are not correlated with velocity in uniform channels. A: All measured instantaneous areas are averaged within their corresponding velocities. Each bar represents a velocity with a range of 0.5 µm/min. B: Velocities with a higher sample size are analyzed in higher detail. Each bar represents a velocity with a range of 0.05 µm/min. No statistically significant differences are observed between groups. N=19, 15, 22, 19, and 28 for 1.5µm, 3µm, 6µm, 12µm, and 2D, respectively. 

found no significant trends in the relationship between instantaneous growth cone areas and velocities (Figure 12). Therefore, altering growth cone area through confinement does not in
itself alter average velocity.

Although overall velocities were not significantly different, the possibility remained that channel width could influence growth cone dynamics. By visually inspecting a randomized handful of growth cone videos, we were able to determine instantaneous velocity thresholds which correlated with phenotypic advancing, pausing, and retracting behavior (Figure 11). Growth cones with velocities exceeding 0.2 µm/min, between 0.2 and -0.2 µm, and below -0.2 µm were considered advancing, pausing, and retracting, respectively. Individual growth cone trajectories were then analyzed to determine the amount of time spent performing each behavior (Figure 13). While certain trends were noticeable, few values were significantly different from others despite major changes in channel width. Growth cones in 1.5 µm channels spent significantly more time pausing than growth cones on unpatterned surfaces. An opposite trend was observed for the percentage of time advancing, though this did not reach statistical significance (Figure 13B). Furthermore, growth cones on unpatterned substrates spent more time retracting than growth cones on 12 µm channels.
Figure 13. Growth cone behavior is altered by channel width. (A) One-dimensional displacement over time of a single growth cone extending along a channel. Circles represent categorization of motion at each point based on velocity. (B) Average of percentage of time spent advancing, pausing, and retracting for growth cones in each group. *p<0.05. N=19, 15, 22, 19, and 28 for 1.5µm, 3µm, 6µm, 12µm, and 2D, respectively. Adapted from Smirnov et al. (2014).
Growth cones of retinal ganglion axons alter both their velocity and shape when they enter the optic chiasm (Mason & Wang, 1997). We therefore sought to evaluate whether

**Figure 14.** Time-lapse taken from a high-time resolution recording of a growth cone exhibiting response to change in confinement. Increases in velocity and area are evident as the growth cone transitions from a 1.5 µm-wide channel to a 10 µm -- wide node.
increased confinement would alter growth cone velocity and shape. We plated CGNs on patterns with 1.5 µm channels interspersed with repeating circular nodes 10 µm in diameter at 60 µm intervals, and imaged growth cones over time as they traversed the patterns. Growth cones in the 1.5 µm-wide region displayed similar behavior to those found in uniform channels: advancing, retracting, and pausing in a seemingly unpredictable manner. However, once growth cones encountered a node, they rapidly accelerated into the node and spread out their filopodia and lamellipodia. Once inside the node, growth cones slowed down and spent time exploring the edges of the node, and ultimately proceeded into the continuing channel (Figure 14).

For each growth cone in every frame, position was recorded relative to the center of the nearest node. A histogram of the data reveals fewer growth cones in the location approaching the node (Figure 15A). In contrast, growth cones are more frequently found inside and following the node. Average instantaneous velocity at each position relative to the node was calculated using individual growth cone displacements between consecutive frames. Growth cones displayed up to a three-fold increase in velocity as they approached and entered the node: between -15 µm and -10 µm from node center, average velocity was 0.34 ± 0.09 µm/min (Figure 15B). Once inside the node, growth cone velocity dropped significantly: between -5 µm and 5 µm from the center of the node, average growth cone velocity was 0.06 ± 0.01 µm/min. After growth cones left the node, their velocity increased to 0.13 ± 0.01 µm/min. This velocity is
significantly lower than in channels with a consistent 1.5 µm width, where growth cones traveled at an average of 0.17 ± 0.03 µm/min. These changes in velocity were accompanied by substantial changes in growth cone area. Preceding and following the node, area hovered around 20-25 µm², similar to that found in the uniform 1.5 µm channels (Figure 15C, Figure 7C). However, as

**Figure 15.** Growth cones respond to local changes in channel width. (A) Histogram of total recorded growth cone locations in all timeframes and growth cones relative to center of node. (B) Average growth cone velocity at each location relative to center of node. (C) Growth cone area averaged at each location measured from center of node. Node is located at -5 to +5 microns. N=19. Adapted from Smirnov et al. (2014).
growth cones entered and explored the node, their area increased to a range of 30-40 \( \mu \text{m}^2 \).

Thus, once inside the node, changes in growth cone area and velocity are inversely correlated.

3.3.4 EFFECTS OF REGULATING NODE SHAPE

Figure 16. Nodes with alternative geometries. Top to bottom: Narrow double-coned nodes, wide double-coned nodes, narrow cone-circle, and wide cone-circle. Scale bar: 5 \( \mu \text{m} \).
In order to better understand the mechanism responsible for the increase in growth cone velocity and area as growth cones enter a node, we fabricated patterns which alternated the shape of interspersing nodes (Figure 16). At first, nodes were designed asymmetrically, with one side exhibiting a short or long conical shape, and the other with a circular shape. This geometry ended up having a polarizing effect on CGNs, as the dominant neurite would often end up traveling into the circular node and extending out of the elongated cone. We therefore also

**Figure 17.** Growth cone size while entering circular versus conical nodes. A: growth cone area while entering a circular node. B: Growth cone length while entering circular node. C: Growth cone area while entering short conical node. D: Growth cone length while entering conical node. All areas are given in $\mu m^2$, while lengths are given in $\mu ms$. 
fabricated patterns with a symmetrical, conical design (Figure 16, top). CGNs were plated on these patterns and observed as previously described.

Growth cones entering either circular or conical nodes were quantified to determine area and length versus position of the leading filopodia and wrist, respectively. Unfortunately, these measurements alone did not produce any obviously distinct results as the variability between growth cones outweighed any significant fluctuations in morphology relative to position (Figure 17). We then attempted to increase the precision of our measurements by using high time-
resolution video capture (5 sec between frames instead of 5 min). Growth cone position relative to the transition region between the lane and node was recorded at the leading edge (tip of protruding filopodia) and the wrist. Growth cones were grouped and their position over time was plotted as they entered either a circular (n=4) or conical (n=5) node (Figure 18). Once again, any differences caused by the change in pattern geometries were outweighed by noise, and a much higher n would have been needed for statistical power in this experiment. We found no difference in area, length, or velocity as growth cones entered either conical or circular nodes.

3.4 DISCUSSION

With the use of micron-scale laser patterning, we engineered substrates that confined CGN growth cones to narrow channels of defined width, allowing high-resolution time-lapse imaging to evaluate the growth and morphology of growth cones over long periods of time as they traversed these channels. Confining growth cones to uniform channels of different widths influences their morphology but not their velocity, suggesting that altering growth cone shape alone does not alter velocity. However, we found that growth cones did respond to changes in channel width: growth cones sped up and then slowed down as they entered nodes interspersing narrow channels. Growth cone morphology was also responsive to decreased confinement, as growth cones rapidly enlarged upon entering a node. Thus, in confinement, growth cone motility appears to be responsive to changes in the environment, but not to the confinement process itself.

We evaluated the outgrowth of isolated CGNs in laminin-coated channels whose width was varied between 1.5 and 12 µm. As previously observed for other cell types (Hammarback et al.,
we found that laminin patterns were very effective in directing and promoting CGN neurite outgrowth, with very few growth cones ever leaving the channels. Average growth cone velocity in uniform channels did not depend on channel width and did not differ significantly from growth rates on a uniform substrate of laminin. Tai and Buettner, on the other hand, found that DRGs on an unpatterned surface extend faster than those in confined lanes (Tai & Buettner, 1998). In contrast to those results, Song and Uhrich found that DRG neurites extend fastest in laminin stripes as compared to an unpatterned surface, but their patterns were not meant to fully confine neurons, and therefore show a significant amount of fasciculation and interaction between axons (Song & Uhrich, 2007). Compared to DRGs, we found CGNs to be ideal for growth cone isolation, since DRG cultures are often confounded by effects of clustering due to a high amount of branching and cell heterogeneity. Our results with CGNs complement previous studies in that our lane widths are capable of reducing growth cone size, allowing us to isolate the influence of growth cone size on extension rate.

Time-lapse imaging revealed that growth cones did not advance steadily: they would pause, advance, and retract. Neurons in 2D environments also exhibit similar behavior (Argiro et al., 1984). In vivo, pausing tends to be associated with decision regions and changes in directionality (Godement et al., 1994; Skaliota et al., 2000). Consequently, one could assume that on confined, linear substrates where growth cones lack choice in directionality, pausing would be unnecessary while forward growth would be more consistent. It is therefore curious that varying channel width proved to have little effect on the percentages of time spent pausing or advancing. Furthermore, growth cones on 2D surfaces actually spent relatively less time pausing than those on 1.5 µm channels. Weigel et al. found that chick spinal cord neurons grown
between elevated ridges exhibited a similar behavior: the percentage of time where velocity was positive remained between 70 and 80% regardless of the inter-ridge distance which growth cones occupied (Weigel et al., 2012). Average positive and negative velocities were also fairly consistent at about 85 and -70 µm/h, respectively. The same methods of analysis applied to our data show that positive velocities were recorded between 60 and 65% of the time, while average positive and negative velocities had a much lower magnitude at 23 and 20 µm/h. These differences in velocity can likely be attributed to cell type and substrate composition.

Growth cone area and aspect ratio were found to vary with channel width. CGN growth cones on wide lanes and 2D unpatterned surfaces had areas on average 1.5 - 2 times larger than those on narrower lanes. Two groups reported similar alterations in DRGs: as compared to growth cones on unpatterned surfaces, growth cone size was reduced in lanes narrower than 20 µm (Clark et al., 1993) and increased in lanes wider than 20µm (Tai & Buettner, 1998). Under some conditions, axon outgrowth is edge-guided (Xing et al., 2010), and it is possible that wider channels drive growth cones to enlarge in order to contact with both edges at once. Although in our experiments average growth cone area was not statistically different between 12 µm and 2D groups, the distribution of all growth cones at all times showed that some growth cones in 12 µm channels were noticeably larger, suggesting that this phenomenon may be present in our system as well. The wide area distributions in each group are likely explained by growth cones’ phasic process of spreading out and contracting their filopodia and lamellipodia (Gallo & Pollack, 1995).
Previous studies have also shown that growth cone height decreases as a result of spreading out on laminin lanes (Messa et al., 2009). In our study, growth cones in wider lanes appeared flatter when examined under SEM (Figure 7B), suggesting a 3D cytoskeletal rearrangement in response to confinement. A more complete evaluation of growth cone volume would have required a long-term fluorescent marker allowing for live, confocal imaging of 3D stacks during growth cone extension. One shortcoming of our patterning system is time investment required to create a single dish – a 2x1 mm pattern often takes up 3 hours. As a result, each experimental session is heavily limited by the number of neurons which can be plated. This limitation makes it virtually impossible to transfect neurons with a fluorescent marker, since the low efficiency would result in too few neurons being labeled. We also tried using a temporary membrane stain (CellMask™), and while it succeeded producing fluorescent growth cones, the effect was short-lived due to internalization and bleaching of the dye as well as phototoxicity caused by constant laser exposure. Ideally, long-term fluorescent imaging could be performed using neurons harvested from a genetically modified mouse, especially one that produces multiple fluorophores to distinguish between different cells (Cai et al., 2013), and intermittent photoexposure using a low-power, low-toxicity wavelength of light.

Growth cone area was also found to change as neurons encountered increases and decreases in channel width. As they entered a node, growth cones both grew larger and slowed down. Féréol et al. observed a similar behavior in DRG growth cones which spread out and paused as they entered a laminin spot, yet remained highly dynamic while supposedly searching for their next target (Féréol et al., 2011). After exiting a node, growth cone area again decreased inside confined channels. This behavior is reminiscent of that seen in retinal ganglion cells:
growth cones tend to be small and streamlined in the optic nerve, but as they reach the optic chiasm, they often become larger and tend to pause (Bovolenta & Mason, 1987; Mason & Wang, 1997). Similarly, Liu et al. found that hippocampal growth cones increased in size when leaving a 5 µm lane and entering an open, 2D region, at which point they were more likely to branch (Liu et al., 2013). It is worth noting, however, that the growth cone area measured in the nodes was equal to that of growth cones continuously confined to channels with a width similar to that of the node, suggesting that the spreading of the growth cone is not dependent on the preceding state of confinement.

A lack of influence by substrate confinement, and thus growth cone area, on extension rate may seem puzzling due to the growth cone’s established role as the primary mechanistic center in axon extension. The most parsimonious explanation is that there exists a feedback mechanism that maintains the overall elongation rate. There are a wide range of factors that can modulate axon growth rate, for example laminin concentration (Marquardt & Willits, 2011; Swindle-Reilly et al., 2012). While larger growth cones might sense more laminin, smaller growth cones might compensate with an alteration in integrin expression to sustain a necessary growth rate, as neurons have been shown to vary their expression levels of integrins based on laminin concentration in the substrate (Condic & Letourneau, 1997).

That there are actually differences in growth cones in lanes of different widths is evidenced by the “jumping” behavior as growth cones encounter a node, suggesting that a sudden change in substrate width disrupts an established steady state equilibrium, resulting in unusually quick growth as more laminin area is encountered in the node. This phenomenon is
similar to the increased rapid movement of DRG growth cones on fibronectin as their filopodia touch laminin (Gomez & Letourneau, 1994). There are several potential explanations for this behavior. The ‘jump’ could be a response to an increased amount of laminin ahead of the growth cone, since receptors on growth cone filopodia influence neurite outgrowth when contacting patterned ECM substrates (Mortimer et al., 2008; Geraldo & Gordon-Weeks, 2009). The high laminin availability does not produce higher growth rates in axons that are continually confined to the wider lanes, presumably because they have already adapted to their environment. Growth cones likely form more point contacts in the node, and may be pulled forward as a result of the increase in force that would arise from a transition to a larger area of traction stresses like those observed in DRG growth cones on uniform 2D substrates (Koch et al., 2012).

A feedback mechanism could also operate through the rate of tubulin polymerization or the organization of microtubules in the neurite shaft, which is tightly regulated, in part through interaction with MAPs (Tymanskyj et al., 2012). For example, growth cones constricted by pattern confinement could upregulate tubulin polymerization rates, either though the removal of capping proteins, an overactivation of +TIPs, or a blockade of severing proteins. When entering a node, the enhanced rate of microtubule polymerization would allow the growth cone to quickly advance and expand, forming multiple stabilized filopodia, as evidenced by our high time-resolution data. An asymmetry in substrate size (lane vs node) could also lead to a higher chance of forward-facing stabilized filopodia. In uniform channels, filopodia and lamellipodia are exposed to an equal amount of surface area both in front of and behind the P domain, while a front-facing asymmetry would bias microtubules to explore filopodia towards the front.
Over repeated high time-resolution trials, we found little evidence that there was a difference between the speeds at which growth cones entered cones versus rounded nodes. One explanation is that the increase in extension rate is not linearly related to changes in substrate size, but works in a step-wise fashion, ramping up past a certain threshold value. If this were the case, one could argue that we simply did not find a low enough threshold value for decreasing the rate at which growth cones jump into nodes. While our data provide some support for this hypothesis, it is unlikely to be true. The rate of branching in hippocampal growth cones as a response to changes in laminin concentration correlates linearly with the degree of laminin concentration change (Liu et al., 2013). Similarly, changes in growth cone velocity are likely to change as a linear function of transition region asymmetry. Unfortunately, as we have shown, growth cone behavior in vitro is not stereotyped, and we likely lacked the necessary sample size to find a significant difference between the conical and circular node datasets.

In order to understand the mechanisms responsible for the modulation of growth cone behavior as a function of channel width, it may be necessary to conduct studies using higher-resolution imaging techniques to identify individual receptors and cytoskeletal proteins. For example, the proposed differences in point contact density between narrow and wide channels could be evaluated in real time using TIRF microscopy (Khalil et al., 2014). Focal adhesions in confined channels have been identified in TIRF by imaging certain proteins, such as vinculin or FAK, and their distributions were compared using fluorescence intensity measurements (Doyle et al., 2009). Therefore, by evaluating the densities of point contacts in confined, wide, and transitional patterns using TIRF, we could test whether growth cones indeed modulate active integrins and point contacts as a response to confinement.
Multiple pharmacologic, genetic, or imaging techniques could be used to investigate the cytoskeletal mechanisms of growth cones on photoablated patterns. For example, in order to examine whether myosin II–fueled retrograde actin flow is responsible for the ‘jump’ behavior seen in growth cones as they enter nodes, the myosin inhibitor blebbistatin could be applied to extending neurons. We expect that since the jump mechanism is likely dependent on engagement of the clutch, neurons lacking myosin II will not be affected by changes in channel width. It is also arguable whether the initiation of the jump is led by point contact formation or microtubule invasion of the forward-facing filopodia. By concurrently observing point contact formation and microtubule plus-end assembly in neurons using high time-resolution fluorescent imaging, one should be able to identify the relative temporal pattern of each event.

Many different patterning techniques have been used to evaluate growth cone dynamics in vitro. Laser-guided patterning has the advantage of producing consistent, micron-scale patterns that can be easily modified to test specific hypotheses. While it has been established that substrate feature size is capable of controlling neuronal outgrowth rates, we have shown that altering growth cone size, per se, will not modulate growth velocity. However, we demonstrated that growth cones do respond to changes in their environment with a change in size and axonal extension rate. In decision regions, this change in growth cone size and velocity has been linked to a change in sensitivity to guidance cues, and we propose that our assay can provide a systematic test of this hypothesis.

4 COMBINING STRUCTURAL, MECHANICAL, AND BIOCHEMICAL CUES
4.1 INTRODUCTION

As we have previously established, the interaction between environmental structure, topography, and molecular cues is important for neuronal growth. Developing neurons follow specific structural pathways provided by scaffolding glia and other adjacent cells, yet the biochemical environment remains an important aspect of their guidance system, steering growth cones away from certain topographies and towards others (Raper et al., 1984). The combinatorial effects of material structure and biochemistry are also important for the design and development of implants used for regeneration in spinal cord injury (Straley et al., 2010). In order to successfully guide neurons through a peripheral and central nerve injuries, multiple implant design strategies have been proposed, including the use of various nanotopographies, aligned fibers, materials of varying stiffness, and biochemically patterned gels (Koh et al., 2010; Reich et al., 2012; Weigel et al., 2012; Gerberich & Bhatia, 2013; Kanno et al., 2014; Prasad et al., 2014). In order to achieve successful regeneration through a glial scar, implants must not only be used to guide neurons through the cavity, but also help bypass the inhibitory cues, such as CSPGs, present in the surrounding environment (Geller & Fawcett, 2002).

Previous observations of growth cones in the optic chiasm have led us to believe that there exists a causal relationship between the biochemical environment and growth cone morphology. The intricate biochemical patterning in the optic chiasm (Table 1) that serves to properly steer neurons towards their appropriate hemispheres is colocalized with the slow progress and large morphology of RGC growth cones. While we have shown that altering growth cone size does not in itself alter velocity in vitro, growth cone response to changes in confinement suggests that
confined growth cones will react differently to an acute change in substrate-bound cues as compared to their non-confined counterparts. Our hypothesis states that larger versus smaller growth cones are more sensitive to the extracellular matrix and other surrounding cues. An increased growth cone size results in a larger surface area of filopodia and lamellipodia, potentially allowing for an increased concentration of surface-bound receptors. Smaller growth cones, on the other hand, may be less sensitive to their surroundings. Therefore, in order to quantify the effect of growth cone morphology on sensitivity to changes in the external environment, we sought to design an approach which would combine growth cone confinement with a selective exposure to additional guidance factors.

While many studies have investigated neuronal responses to a structural or biochemical environment, few have attempted a combined approach in order to address the synergistic or antagonistic effects of these guidance mechanisms. As previously described, a pattern of repeating pillars was shown to enhance the attractive effects which netrin-1 exerted on extending hippocampal neurites, while eliminating the repulsive effects of both netrin-1 (which, the reader may recall, is bifunctional) and Semaphorin3A (Kundu et al., 2013). In PC12 cells, decreasing groove width was shown to cooperatively enhance NGF-mediated neurite formation, while certain geometries removed the necessity for NGF altogether (Foley et al., 2005). Finally, DRG alignment was aided by increased laminin concentration on grooves of select widths (Miller et al., 2002). Outside of those examples, little evidence has been collected showing the effect of cooperative directional cues. In the following sections, we examine a few distinct approaches which could potentially be used understand the relationship between growth cone morphology and its sensitivity to the biochemical and mechanical environment.
4.2 METHODS

**ABLATED PATTERN FABRICATION.** Photoablated patterns were created using methods described in section 3.2.

**LAMININ LABELING AND ATTACHMENT.** Laminin was conjugated with Alexa Fluor-488 dye (Life Technologies) using supplied protocols. Fluorescently labeled laminin was diluted to 5 µg/ml in PBS with 0.1% Pluronic F-127 (Life Technologies) and added to 2D or photoablated coverslips for 30 min at room temp. Dishes were washed 3x with PBS and kept in PBS until use.

**MICROCONTACT PRINTING.** Previously published protocols were adapted for µCP (Théry & Piel, 2009). To create a silicon master, 3” silicon wafers were cleaned in piranha solution for 20 min and baked for 5 min at 200°C for dehydration. A 10 µm layer of SU-8 5 photoresist was deposited onto the wafer by spincoating. Spincoating involved a 500rpm ramp at 100 rpm/s, holding for 5 s, and then ramping up to 1300RPM at 300 rpm/s for 30s. Wafers were bakes at 65°C for 1 min and at 95°C for 2 min. SU-8 was then exposed to UV using a mask aligner (Karl Suss) and developed for 2 min with agitation in SU-8 developer. The master was then rinsed with isopropyl alcohol and DI H₂O and baked for 15 min at 200°C. In order to prevent adhesion between the master and PDMS, masters were silazined using vapor deposition of tridecafluoro-1.1.2.2-tetrahydrooctyl trichlorosilane in a vacuum desiccator for at least 1 hour.

Sylgard 184 and curing agent were mixed at a 10:1 ratio by weight and bubbles were removed by 10 min exposure in a vacuum chamber or centrifugation. A slow-pouring apparatus was set up to work inside a large vacuum chamber in order to pour the mixed PDMS solution
onto the silicon master without the presence of air (which could lead to bubble formation). The PDMS was then cured overnight at 65°C.

Immediately prior to μCP, glass-bottom dishes and PDMS stamps were made hydrophilic using a 3 min exposure to O₂ Plasma in a Plasmalab 80 Plus (Oxford Plasma). Stamps were soaked with 25 µg/ml 488-laminin or 200 µg/ml CSPG with Texas Red for 30 min, then washed 3x with PBS and H₂O. Stamps were then dried completely. Laminin stamps were placed face-down on hydrophilic coverslips and held down with a 50 g weight for 10 min. Laminin stamps were removed and replaced with CSPG stamps, facing perpendicular to laminin patterns. CSPG stamps were removed after 10 min and surfaces washed 3x with PBS.

**POLYACRYLAMIDE GEL FABRICATION.** Polyacrylamide gels were prepared according to published procedures (Koch *et al.*, 2012). Briefly, 20 mm glass-bottomed dishes were coated with 2 mg/cm² CellTak (BD Biosciences). Then 18 mm cover glass slips were coated with Sigmacote (Sigma-Aldrich) to make nonadhesive top coverslips. Acrylamide and bis-acrylamide (Bio-Rad Laboratories, Richmond, CA) were mixed in PBS solution to a final volume of 1 ml at appropriate concentrations to achieve the desired gel stiffness determined by previous rheological tuning (Koch *et al.*, 2012). A FluoSphere bead solution (0.2 mm, 660 nm; Invitrogen) was added at 5% volume. The final solution was degassed for 15 min and put on ice for 5 min. Polymerization was initiated by addition of 10 ml of freshly prepared ammonium persulfate (10% w/v solution; Sigma-Aldrich) and 3 ml of N,N,N,N-tetramethylethylenediamine (TEMED; AcrosOrganics, Morris Plains, NJ). Immediately after initiation, 5 ml of polyacrylamide solution was pipetted onto the MatTek dish coverglass and the nonadhesive top coverslip was quickly
placed onto the gel droplet and gently pressed down. The dish was inverted to facilitate settling of fluorescent beads at the upper gel surface. After 30 min the gel was immersed in water for 10 min, and then the top coverslips were gently removed under water. The gels were allowed to swell in dH₂O for 1–2 h before surface coating treatment. Gels were coated with 2 mg/cm² CellTak (BD Biosciences) for 20 min.

**SOFT SUBSTRATE PATTERNING (MEMPAT).** Patterns were created on soft Polyacrylamide gels using a modified procedure described (Wang et al., 2002). A SU-8 master with raised features on a silicon wafer was created using methods described for μCP. A standard PDMS mixture was spun onto the wafer to create a layer of PDMS about 9 μm thick, or 10% less thick than the SU-8 feature layer. The PDMS was then cured for 1 hour at 90°C, and thicker edges were ‘painted’ on the outside of the pattern to allow for easier membrane removal. Membranes were then cut out, peeled off, and exposed to 1.5 min of oxygen plasma using Deep Reaction Ion Etching. Membranes were placed on Cell-Tak coated polyacrylamide gels and allowed to dry. A 25 μg/ml solution of laminin was applied to the top of the membrane for 30 min, and rinsed off 3x with PBS. The gels are then immersed in PBS and the membrane peeled off.

**SOFT SUBSTRATE PATTERNING (PVA FILM TRANSFER).** PVA transfer patterning onto soft polyacrylamide gels was performed by adapting previously published protocols (Yu et al., 2012). PVA was dissolved to 5% in 20ml H₂O at 90°C, poured into a 150 mm petri dish, and left to dry for 2 days at RT. The remaining film was then removed using tweezers and cut by a scalpel into 10 mm-wide circles. μCP stamps were inked with 10% 488-BSA or 25 μg/ml laminin, washed 3x with PBS, dried, and placed face-down on PVA films for 10 min with a 50 g weight.
Polyacrylamide gels were desiccated for 10 min at 65°C to remove any liquids present on the surface without allowing crystallization of the gel. PVA films were placed face-down on the gels and slightly pressed down using tweezers to ensure direct surface interaction. After 30 min, PBS was used to rinse and wash away PVA films.

**STREPTAVIDIN-BIOTIN SPOT BINDING ASSAY.** Biotin labeling of CSPGs was done using supplied manufacturer protocols. The presence of a chromophore on each biotin molecule in the Chromalink™ biotin labeling kit (Solulink) allowed us to quantify the amount of biotin molecules attached to each molecule of CSPG with UV chromatography. By trying multiple concentrations and using Laminin as a control, we were able to identify optimal conditions for labeling (Table 2).

Streptavidin was conjugated using Alexa Fluor-568 dye (Life Technologies) using supplied protocols. Streptavidin was diluted by 1:10, 1:100, and 1:1000 in H$_2$O and applied in 2 µl quantities to either 2D glass or photoablated pattern for 1 min at RT. A biotin-CSPG spot was used as a control. Coverslips were then washed 3x with H$_2$O. Coverslips were then immersed with a solution of 50 µg/ml Biotinylated CSPGs in PBS and incubated for 30 min at 37°C. Dishes were washed 3x with PBS. CSPGs were visualized by staining with 1:200 CS-56.

**CSPG SPOT ASSAY.** An interface between laminin and CSPGs was created by placing a 1 µl drop of chicken CSPG (Millipore) with Texas Red in the center of a laminin-coated coverslip or edge of a laminin-coated pattern, in order to achieve about 50% coverage of each channel. Texas Red was used to visualize the interface and was used alone for negative control experiments. CSPG adhesion was again confirmed by staining with 1:200 CS-56 antibody (Abcam).
**CELL CULTURE.** Cultures of dissociated mouse CGNs were prepared from postnatal 5-day-old C57BL/6 mice and plated as described in section 3.2. Cultures of dissociated DRG neurons were prepared from P0-P1 rat pups as described previously in (Koch et al., 2012). DRGs were removed from the lumbar region rat pups, trimmed, washed in Dulbecco’s modified Eagle’s medium (DMEM), and enzymatically digested for 20 min in 3 ml 0.25% trypsin with 10 mg/ml DNase/Ca²⁺ and Mg²⁺-free Hanks balanced salt solution (HBSS). Explants were then dissociated by trituration with a fire-polished Pasteur pipette. The reaction was stopped by addition of an equal volume of fetal bovine serum (FBS), followed by addition of DMEM to a final volume of 15 ml. Cells were then pelleted, resuspended in 5 ml DMEM, and passed through a 100 mm cell strainer. The cell strainer was rinsed twice with 5 ml DMEM and the cell solution was pooled, pelleted, washed twice in DMEM, and finally resuspended in Neurobasal media (Life Technologies, Grand Island, NY). DRGs were cultured in Neurobasal medium with 2% B27, 5% horse serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml Fungizone, and 10 mM HEPES, and 2 nM NGF. All cell culture materials were obtained from Gibco (Grand Island, NY). Cells were plated at relatively low densities of 1 × 10⁴ cells/dish and incubated at 37°C and 5% CO² atmosphere. Glia cell numbers were kept low by careful trimming during the dissection process and using a low plating density. In addition, single neurons/growth cones were selected for observations to avoid interference from glia cells or other neurons.

**IMAGING AND ANALYSIS.** Phase contrast images of CGNs were obtained using a Nikon Eclipse TE2000-E inverted microscope equipped with a 37°C and 5% CO² incubation chamber and a motorized stage (Prior Scientific). Metamorph software (Molecular Devices) with the multidimensional acquisition plugin was used to capture images and control all hardware.
Images were acquired using a 20x objective and digital camera (Hamamatsu Photonics). Time-lapse brightfield images of DRGs were taken on a TE2000-U inverted microscope (Nikon) equipped with a Hamamatsu camera and 10x objective. Cell viability on the microscope stage was ensured by means of a live cell chamber equipped with an objective heater (Tokai Hit, Shizuoka-Ken, Japan) that controlled temperature and pH. Protein fluorescence was detected using epifluorescence illumination with a mercury lamp, a 488 nm and a 568 nm filter cube. Image processing was performed using ImageJ (Schneider et al., 2012).

4.3 BIOCHEMICAL BARRIERS ON PHOTOABLATED PATTERNS

After having shown the ability of narrow photoablated channels to alter growth cone size through confinement, we sought to pair parallel channels of different widths with an inhibitory biochemical boundary. With laminin established as a successful permissive cue, we chose to incorporate CSPGs as a model for biochemical inhibition. Previous work in our lab has shown that PC12 cells are sensitive to CSPGs, and, when plated on PLL, will avoid entering a CSPG-rich zone by redirecting their growth in a different direction (Kelly et al., 2010). Predictably, CGNs exhibit this effect as well, avoiding CSPGs in a concentration-dependent manner.

If our prediction that an increased growth cone size results in greater biochemical sensitivity holds true, growth cones in wider channels would have a lower CSPG concentration threshold for stalling than those in narrow channels. Growth cones in narrow channels would therefore be more likely to cross into region of surface-bound CSPGs when the CSPG concentration is held constant. In contrast to previous turning assays which have relied on 2D environments, the behavior of confined growth cones encountering CSPGs would block their
ability to turn, likely leading to other interesting behaviors. In fact, it is quite possible that confined growth cones facing an inhibitory barrier would turn dystrophic, leading to a new model equipped to further study this behavior which has, to our knowledge, rarely been reproduced \textit{in vitro} (Tom \textit{et al.}, 2004).

The creation of CSPG barriers on photoablated channels would require a novel modification of the patterning approach. When initially developing µPP, Andrew Doyle was able to achieve the adhesion of multiple surface proteins through repeated rounds of ablation and protein coating (Doyle, 2009). First, a protein pattern would be prepared using methods similar

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure19.png}
\caption{Serial ablation with µPP to generate patterns of multiple proteins – Fibrinogen (green), vitronectin (red), and fibronectin (blue). DIC image shown on bottom left. Scale bar is 5 µm. Adapted from (Doyle \textit{et al.}, 2009).}
\end{figure}
to those described earlier; a glass-bottom dish would be activated, coated with PVA, ablated, quenched, and coated with an ECM protein. Next, the process of adding other proteins required blocking the surface with BSA, drying out the pattern with ethanol, and ablating a new, non-overlapping pattern to serve as an attachment point for the second ECM molecule. Since the previously pattern had been blocked with BSA, the secondary protein would refrain from adhering to the same region. This process could be repeated multiple times to create a pattern of adjacent yet isolated proteins (Figure 19). Unfortunately, while well-suited for some applications, this method was of no use to our approach since it does not allow for the adhesion of multiple overlapping proteins.

<table>
<thead>
<tr>
<th>Initial Biotin Concentration</th>
<th>Incubation</th>
<th>Molecule</th>
<th>RESULT - Biotins per protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x</td>
<td>120 min</td>
<td>CSPG</td>
<td>18.8</td>
</tr>
<tr>
<td>50x</td>
<td>120 min</td>
<td>CSPG</td>
<td>125</td>
</tr>
<tr>
<td>1x</td>
<td>120 min, then overnight at 4°C</td>
<td>CSPG</td>
<td>18.4</td>
</tr>
<tr>
<td>50x</td>
<td>120 min, then overnight at 4°C</td>
<td>CSPG</td>
<td>132</td>
</tr>
<tr>
<td>1x</td>
<td>120 min</td>
<td>LAMININ</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 2. Results from biotin labeling of CSPGs using Chromalink™ biotin labeling kit.

4.3.1 RESULTS AND DISCUSSION
Figure 20. CSPG adheres poorly to patterns. Texas Red signal, colocalized with CSPGs, was used to identify CSPG presence. A: CSPG forms clear boundary on non-patterned, exposed 2D glass surface. B: A weak fluorescent signal is present outside of ablated lanes, but no evidence of CSPG within lanes exists. C: CSPG applied through soaked filter paper.
We decided that the most straightforward approach for creating a CSPG barrier on confined lanes would be through the use of the established spot assay, adapted for an ablated pattern. Previously, a 5 µl drop of chicken CSPG diluted in HBSS with Texas Red would be placed on a coverslip previously coated with 200 µg/ml PLL and incubated for 30 min at 37°C in a humid chamber. The spot would then be washed away with HBSS and cells plated on the coverslip. Since we had determined that laminin did not require PLL to attach to photoablated lanes, we attempted to replicate a CSPG spot assay on a pattern of photoablated, parallel lanes. Using a 10x light microscope, a 2 µl spot of 50 µg/ml CSPG was applied to one side of an ablated region, resulting in around 50% coverage of the parallel channels. The spot was incubated for only 10 min, since any more time resulted in too much condensation collecting on the hydrophilic PVA, leading to unwanted dilution and spreading of the CSPG spot. Unfortunately, CSPG adhesion to the exposed glass was poor, as illustrated by the Texas Red signal in Figure 20B. The Texas Red signal was later verified using CS-56 antibody labeling (data not shown). We also attempted to localize CSPG adhesion by first soaking small strips of filter paper in CSPG solution, and then applying them perpendicular to the pattern, but the resulting CSPG concentration was highly aberrant and direct contact with the filter paper caused deformations in the PVA pattern (Figure 20C).
We next attempted to use a streptavidin-biotin interaction to improve CSPG adhesion. Biotin, an egg-white protein, and streptavidin, its bacterial counterpart, are standard reagents used for diverse protein labeling, detection, and surface adhesion schemes (Wilchek & Bayer, 1990). In previously studies, researchers have successfully conjugated chondroitin sulfate GAG

**Figure 21.** Biotinylated CSPG (green) does not bind to streptavidin (red). Left: Streptavidin spot incubated with biotinylated CSPG. CS-56 antibody staining indicates that CSPG is more prevalent outside of streptavidin spot. Right: Biotinylated CSPG spot without streptavidin present.
chains to biotin, although our literature search did not reveal any attempts at biotinylating entire CSPG molecules (Deepa et al., 2002; Ando et al., 2010). If successful, we would be able to create a spot of Streptavidin on a patterned surface and flood the surface with biotinylated CSPGs, forming a localized spot consisting of Streptavidin-Biotin-CSPG complexes. Biotinylation of CSPGs

**Figure 22.** A coating of at least 0.05 µg/ml PLL will promote cell adhesion to PVA. 10x phase contrast images show CGN cell bodies on a PVA surface 1 day following plating.
was successful, resulting in about 18 biotin molecules per CSPG protein (Table 2). Unfortunately, biotinylated CSPGs did not colocalize with Streptavidin spots, and bound competitively to glass surfaces (Figure 21).

It is difficult to identify the reasons behind CSPGs’ apparent reluctance to functionally bind to photoablated glass, as well as to form biotin-streptavidin complexes. One possible justification for these behaviors is the unusual size and shape of the CSPG molecule. CSPGs are relatively large, ranging between molecular weights of 250 and 400 kD. Furthermore, the molecules contain a protein core which is surrounded by an often dense meshwork of highly sulfated GAG chains. The sulfate groups between these chains give CSPGs a highly negative charge. Since the Biotinylation process likely adds biotin molecules to the protein core, the streptavidin-biotin bond would need to break through the highly negative GAG structure. At pH=7, streptavidin is also negative, and therefore would likely repel the CSPG molecule before attaching to its biotin residues. The negative charge on CSPGs may also be responsible for its inability to bind patterned channels, since the photoablated glass likely holds a negative charge as well. We therefore tested whether CSPG binding to ablated channels could be enhanced by using a lower pH or the addition of a positive amino-acid such as lysine, but neither process had any appreciable effect (data not shown).

On 2D glass, the adhesion of proteins and cells is easily achieved by an initial PLL coating step. Unfortunately, the high polyvalency of PLL initially prohibited us from incorporating it into our photoablated patterns. At its typically applied concentration of 200 µg/ml, PLL would undo the non-adhesive properties of PVA, allowing cells to adhere outside of the ablated zone. By
titrating the concentration of PLL coating down and testing cell adhesion on PVA-coated dishes, we determined that the concentration of PLL needed to be lower than 0.05 µg/ml in order for PVA to retain its non-adhesive properties (Figure 22). Next, we created CSPG spots using a solvent of 0.01 µg/ml PLL in H₂O, and determined that this does, in fact, allow for CSPG protein deposition on an ablated pattern (Figure 23). While these results are promising, the roughness of
the fluorescent border on patterned versus 2D surfaces indicated that further optimization may be required before testing the effects of confinement on CGN sensitivity to CSPGs. Furthermore, the disparities between CSPG adhesion to ablated glass versus 2D, unpatterned coverslips suggests that the latter may not be an ideal control for cell outgrowth studies, and quantification of CSPG surface concentration via radiolabeling (Snow et al., 2002) on ablated versus 2D surfaces

**Figure 24.** Laminin and CSPG patterns resulting from photolithographic techniques. A: Intersecting lanes of laminin and CSPG achieved with μPP. While laminin lanes are contiguous, CSPG lanes are segmented. B: Printed lanes of laminin intersected by CSPG speed bump created with microfluidics. C,D: CSPG and Laminin interface created by spot assay.
may be necessary. Conversely, proper 2D controls may be designed by ablating away much larger regions of PVA, although this approach may likely be significantly more time-consuming.

### 4.4 PHOTOLITHOGRAPHIC APPROACHES

In addition to using µPP, we also explored photolithographic techniques for the purpose of creating inhibitory boundaries for neurons extending on guided channels. When fabricating patterns for neuronal guidance, laminin is typically printed in either continuous lanes, closed networks, or intermittent spots (Tai & Buettner, 1998; Vogt et al., 2003; Ruiz et al., 2008). The ease of pattern fabrication makes techniques such as µCP and microfluidics appealing, yet few studies have attempted to create patterns of overlapping molecular layers.

#### 4.4.1 RESULTS AND DISCUSSION

In order to combine both laminin and CSPG signals, we tested patterns consisting of microcontact-printed laminin lanes which overlapped with either CSPG ‘speed bumps’ created by µCP or microfluidics, or a CSPG (Figure 24). The process of printing intersecting laminin and CSPG lanes often proved difficult and inconsistent. The overlapping region would often only have one of the cues present, as evidenced in Figure 24A; if laminin was stamped down first, then it would form continuous lanes, while a second stamp, carrying CSPG molecules, would often create segmented lanes characterized by breaks at every laminin interface. The reason for this biochemical interference is likely due to a weak affinity between CSPGs and laminin – while CSPGs are capable of binding to glass, they resist direct attachment to laminin molecules. It has been previously reported that a monolayer of laminin formed by µCP is around 15 nm high, and
forms a meshwork with 100 nm pores (Sgarbi et al., 2004). Such a monolayer would make it exceedingly difficult for CSPGs to bind directly to glass, and is likely the reason for the segmented lane effect.

Since μCP relies on desiccated proteins, we hypothesized that overlapping CSPG and laminin regions could be better achieved by immobilizing CSPGs from solution. We repurposed our μCP stamps into microfluidic devices by inverting them perpendicular to previously stamped laminin lanes, and immersed one end with a solution of CSPGs. Capillary action drove the CSPGs through the stamp, and lanes of CSPG were formed overlapping with laminin (Figure 24B). While the molecular overlap was not always complete, this method was more successful for colocalizing proteins than repeated printing. Furthermore, we were surprised to learn that the attachment of a microfluidic device to a glass coverslip did not distort the underlying laminin pattern.

While unable to create localized strips of CSPG, a spot assay was also successful in forming an intersecting laminin and CSPG zone (Figure 24C, D). Once laminin lanes were created by μCP, a 5 μl drop of diluted CSPGs was added to the pattern and incubated for 30 min. While the spot formed a distinct boundary, it was unpredictably affected by the underlying laminin pattern, resulting in isolated concentration gradients near the spot’s edge (Figure 24C).

Since we did not have access to the tools required to make patterns with features smaller than 10 μm, we chose to use DRG neurons due to their relatively larger size compared to CGNs. DRG cultures are generally a more heterogeneous than those of CGNs, making them unfit for μPP experiments due to constant interference between neurons and glia, making it difficult to
find isolated growth cones. µCP, on the other hand, allowed us to create much larger patterns in a smaller amount of time, so the chance of finding isolated growth cones remained plausible.

Similar to previous studies, we found that DRGs had the capacity to extend along printed laminin lanes (Figure 25). Furthermore, our preliminary results indicated that growth cones on wider lanes were less capable of passing through CSPG ‘speed bumps,’ than those on narrow lanes (Figure 25). These findings supported our hypothesis that growth cones in confined lanes would be less sensitive to inhibitory biochemical cues than those traveling in 2D. Growth cones on wider lanes also tended to branch when encountering CSPG interfaces. A similar observation was
made in hippocampal neurites which remained isolated when confined to a narrow channel, but immediately branched when exposed to a change in laminin concentration, regardless of whether the concentration was increased or decreased (Liu et al., 2013). Neurite branching is preceded by a decrease in growth cone velocity, and may therefore be mechanistically similar to an increase in growth cone complexity and size seen in decision regions such as the optic chiasm or a photoablated node. Therefore, a suppression of branching behavior in confined lanes may be partly responsible for allowing neurons to more easily transverse a CSPG boundary.

When patterned overlapping biochemical cues, it is important to keep in mind that the surface configuration of the molecular layers will likely affect the relative bioactivity of the molecules. In 2011, Theilacker et al. found that when laminin is deposited via microcontact printing onto fibronectin deposited from solution, the bioactivity of the underlying fibronectin is blocked by 84% (Theilacker et al., 2011). Printing of fibronectin into laminin, on the other hand, reduces laminin bioactivity by a mere 27%, suggesting that the steric interference is specific to molecular structure (Theilacker et al., 2011). A similar effect may be present when CSPGs are deposited onto laminin, decreasing the overall functionality of laminin by physically blocking it from cell surface receptors. In fact, certain CSPGs have been shown to interact directly with laminin, fibronectin, and other ECM proteins (Rhodes & Fawcett, 2004).

4.5 CONFINEMENT ON SOFT SUBSTRATES

In addition to structural and biochemical guidance, growth cones encounter environments with varying mechanical properties established by the cytoarchitecture of the surrounding ECM and adjacent cells (Franze & Guck, 2010). Neurones employ mechanical forces to aid in neurite
elongation and guidance, and the growth cone itself is responsible for active force generation (Suter & Miller, 2011). In fact, neurons may be guided by exposure to substrates of varying stiffness. When extending on 3D collagen gels with gradual changes in stiffness, DRG neurons grew significantly longer down rather than up the stiffness gradient (Sundararaghavan et al., 2009). Furthermore, substrate stiffness can affect neuronal sensitivity to biochemical cues, including laminin and fibronectin, and has a direct effect on the expression levels of focal adhesion-related proteins such as pPKCα and pFAK (Marquardt & Willits, 2011; Chen et al., 2013).

Little is known regarding the mechanisms responsible for growth cone guidance by substrate rigidity. It is generally accepted that by forming point contacts with the ECM, growth cones transfer intracellular tension to the extracellular environment, literally pulling on the substrate during advance (Suter & Miller, 2011). This durotaxic behavior is specific to neuronal type: DRGs have shown a preference for specific substrate stiffness values, while no such preference was found in hippocampal neurons (Koch et al., 2012). The differences between DRG and hippocampal neuron interaction with the external environment likely has to do with their respective sizes and point contact distribution; DRG growth cones are much larger and have a more dense distribution of point contact-related proteins than their hippocampal counterparts (Koch et al., 2012). Since confinement has the ability to modulate growth cone size and the distribution of laminin available for point contact formation, we attempted to develop an interface between structural and mechanical guidance for neurite outgrowth.
Protein patterning has previously been achieved on soft substrates, yet we found no data indicating whether the established methods were applicable to materials with neuron-relevant rigidity. Substrate stiffness is typically rated by elastic modulus; for example, the elastic moduli of bone, cardiac muscle, and CNS tissue are about 15 GPa, 100 KPa, and less than 1 KPa, respectively (Rho et al., 1993; Mathur et al., 2001; Lu et al., 2006). Substrate stiffness in vitro can be controlled by modifying the amount of crosslinking of various soft materials, such as PDMS or polyacrylamide (Koch et al., 2012; Chen et al., 2013), but the addition of a patterning step makes the process much more complicated. Since µCP requires that a topographic stamp be placed on a pattern, the softest substrate which has been used without becoming deformed had an elastic modulus of 5 KPa (Palchesko et al., 2012). We therefore attempted to adopt two other techniques have been developed in order to pattern substrates softer than 5 KPa. The first was a membrane patterning technique (MEMPAT) which incorporates the use of a thin PDMS membrane fabricated using photolithography (Wang et al., 2002), while the second relied on a thin, dissolvable PVA film which acted as a proxy between a µCP stamp and the final substrate (Yu et al., 2012).
Figure 26. Membrane patterning of laminin segments on a polyacrylamide gel. A: SEM view of the top of a 10 µm-wide wall on a silicon master. B: 45° SEM views of a PDMS film being removed from a wall, forming a hole in its place. C: 488-laminin (green) left behind on a polyacrylamide gel after removal of PDMS membrane. D: Fluorescent beads (red) indicate deformations in gel surface.
4.5.1 RESULTS AND DISCUSSION

Using a photolithographic process similar to that for creating µCP stamps, we created a silicon master populated by raised 10 µm-wide walls which would serve as perforation points in a PDMS membrane (Figure 26A). A thin layer of PDMS was deposited onto the master through spincoating and then peeled off when ready for use (Figure 26B). Patterning was achieved by then placing the membrane on a soft polyacrylamide gel with a stiffness of 1 KPa and allowing the gel and membrane to dry. The gel would then be immersed with a laminin solution, but only the regions exposed by the holes in the membrane would be exposed to the protein, creating a localized strip of laminin on the surface of the gel (Figure 26C). We found a major limitation of this process to be the lack of choices which we had in controlling feature size; specifically, that we were unable to fabricate long lanes of laminin similar to those created by µCP or µPP. While these thin membranes could very well be used to create arrays of circles or similar symmetrical shapes (Ostuni et al., 2000; Wang et al., 2002), they deformed easily and were nearly impossible to control when dealing with elongated features. Furthermore, we found that the process disrupted the uniformity of the gel surface (Figure 26D). These distortions were likely either due to the drying of the polyacrylamide prior to laminin deposition or stretching as the membrane was peeled off of the gel.

In order to address the shortcomings of the MEMPAT process, we chose to try a different technique for creating confined biochemical patterns on soft, 1 KPa substrates. In 2012, Yu et al. showed that using µCP, a pattern of collagen lanes could be printed onto a thin film of PVA, and then transferred to a soft polyacrylamide gel. In theory, the PVA film could be placed directly on
the soft gel and then dissolved with PBS, leaving behind the printed ECM pattern. We performed the PVA film transfer using μCP stamps inked with either fluorescently labeled BSA or laminin. Once the films were immersed with PBS, they did not dissolve, but immediately expanded in size and, after multiple washes, floated away from the gel surface. After the transfer process,

**Figure 27.** Protein patterns created by PVA film transfer method on 1 KPa polyacrylamide gel with fluorescent (red) beads. A: 488-BSA lanes (green) on cracked polyacrylamide gel. B: 488-Laminin lane (green) on polyacrylamide gel. (red).
imaging revealed that patterns had been distorted during the process, and the gels were often cracked or torn (Figure 27).

The distortions and cracks in the patterns following PVA film transfer likely resulted from the film’s swelling while it was still attached to the desiccated gel. We found that PVA film did not dissolve easily, but would swell immediately and significantly when exposed to water. While it is possible that the original authors used a different kind of PVA, which can vary between vendors by molecular weight or level of hydrolysis, these factors were not specified in their methods.

Since the both the PDMS membrane and the PVA film had to make direct contact with the gel, we found that the gel had to be fully desiccated before pattern transfer, potentially resulting in a serious miscalculation in rigidity by the previous authors of these methods. The substrate stiffness of polyacrylamide gels is usually assumed based on results from known concentrations of gel ingredients, such as acrylamide and its polymerizing agents. In our experience, we have found that soft gels are extremely sensitive to external factors including humidity and physical contact. It is therefore presumptuous to assume that stiffness will remain consistent in gels which are completely dried out, physically touched, and then rehydrated. We suggest that gel stiffness should be assessed by post-patterning quantification with an AFM (which, unfortunately, was unavailable to us during the timing of these experiments).

In conclusion, we found that while patterning gels with a stiffness lower than 1 KPa was possible, there were multiple technical issues which had to be overcome in order to create reproducible patterns on gels with a predictable and consistent stiffness.
In this study, we sought to (1) establish a model system to control neuronal growth cone morphology in a reproducible way, (2) test whether altering growth cone morphology would lead to changes in growth cone velocity, and (3) create an assay which would allow us to test whether growth cone morphology affects sensitivity to inhibitory chemical cues. We successfully accomplished our first aim by confining CGNs to narrow, laminin-coated channels surrounded by a non-permissive region of PVA. The channels were creating by selectively ablating away regions of PVA using a two-photon laser, and then coating the exposed glass through soluble deposition. CGNs remained adherent to the confines of the lanes, and were directly observed using phase microscopy. As per our prediction, we found that neurons on narrow channels (1.5 – 6 µm) had smaller, narrower growth cones than those extending on wide, 12 µm or 2D, unpatterned substrates.

We also found that growth cone morphology does not directly affect growth cone velocity. Average growth cone velocity was widely distributed, and did not change significantly as an effect of channel width. Instantaneous velocities, as well as distributions of advancing, pausing, and retracting behaviors, also remained relatively unaffected by changes in channel width and growth cone area. However, we did find that growth cone velocity changed dramatically as an effect of changes in channel width. Specifically, growth cones extending on narrow, 1.5 µm channels would increase their speed as they entered a 10 µm - wide node. Once inside the node, growth cone velocity would decrease, while their area would increase. We attempted to further dissect the mechanisms behind these velocity changes by altering the
shape of the transition region between the confined lane and wide node, but did not find any differences in velocity. We suggested that the slowing down and increase in size is reminiscent of RGC growth cones traveling through the optic chiasm, although the mechanisms behind these behaviors may be different. We also suggested that the lack of change in velocity as a response to changes in uniform channel width may be due to an intrinsic homeostatic mechanism within the growth cone which serves to regulate velocity by compensating for various extracellular factors. While such a mechanism would serve to equalize velocities in uniform channels, it may contribute to the abrupt changes in behavior as growth cones were exposed to changes in channel width, as seen in non-uniform lane/node patterns.

Finally, we examined multiple approaches to testing the combined effects of biochemical, structural, and mechanical signals on neuronal outgrowth. We ultimately found that two patterning techniques which were claimed to be applicable on soft substrates were not capable of creating patterns without significantly deforming the substrate. Both membrane patterning and μCP transfer using PVA film ended up either tearing or otherwise damaging the soft gels, leading us to the conclusion that these techniques are poorly suited for patterning substrates softer than 1 kPa.

In order to determine whether growth cone morphology has an effect on sensitivity to inhibitory guidance cues, we attempted to engineer an inhibitory region of CSPGs overlapping with photoablated lanes of varying widths. In theory, this approach would create a boundary within individual channels between a permissive, laminin-coated and inhibitory, CSPG-coated surface. Once plated, CGNs would alter their morphology based on channel width, and we would
measure whether channel width has any effect on the growth cone’s ability to enter the inhibitory CSPG zone. We predicted that smaller, streamlined growth cones would be less sensitive to the CSPGs, and would therefore be more likely to enter the inhibitory regions than their larger counterparts. Unfortunately, we found that CSPG deposition onto photoablated surfaces was quite difficult. Unlike laminin, CSPGs did not adhere to the exposed glass without any secondary treatment. We attempted to use a biotin-streptavidin linker complex to aid in surface adhesion, but biotinylated CSPGs remained resistant to colocalizing with streptavidin. We suspect that this unusual behavior is a result of CSPGs’ unique structure, characterized by a protein core surrounded by highly sulfated glycosaminoglycan (GAG) chains. This sulfation is responsible for a highly negative overall charge on the CSPG molecule, likely repelling it from a negatively charged ablated glass surface. Furthermore, since biotin is attached to proteins via a succinimidyl ester which binds primarily to lysine residues, it likely localizes to the CSPG protein core and is blocked from streptavidin adhesion by GAGs. We ultimately chose to use a low concentration of PLL to promote CSPG adhesion to ablated lanes, although we did not have a chance to fully test this assay on neurite extension. We did find, however, that some neurites extending in narrow versus wide µCP laminin lanes were more likely to travel past perpendicular CSPG regions, suggesting that a reduction in growth cone size induced by confinement may make growth cones less sensitive to inhibitory cues.

6 CONCLUSION

We fabricated substrates consisting of narrow, laminin-coated regions surrounded by non-adhesive PVA in order to control neuronal growth cone morphology through confinement.
Indeed, confining CGN growth cones proved to decrease their area, although the velocity and distributions of movement behaviors remained relatively unchanged. When growth cones were exposed to non-uniform lanes and encountered an increase in confinement, they temporarily and drastically increased their velocity and area. We also investigated multiple approaches to combine growth cone confinement with either biochemical or mechanical cues, and found that multiple published patterning techniques for soft substrates did not translate well to CNS-level stiffness levels. Finally, we were able to collect preliminary data indicating that confined growth cones may be less sensitive to inhibitory biochemical cues.


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