Leading the Blind: *Guidance of Neuronal and Vascular Growth*

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Summary

Accurate neuronal and vascular growth during development is critical for an organism's function and survival. Neurons, however, would not grow into a spinal cord, nor would endothelial cells form vascular tissue, if not for molecular guidance signals. These cells are blind to the fate of their growth. Two decades ago, my lab, with numerous collaborators, began the process of elucidating multiple chemotropic proteins expressed in mouse models of the developing nervous system. Their presence selectively attracts and repels axons to guide their growth. These chemotropic factors play dual roles depending on the receptor to which they bind and the interactions between these receptors. In tracking the unique growth pathway of commissural axons, we characterized the activity of five receptor-ligand complexes. We showed neuron attraction was mediated by DCC-Netrin and Rig-1-Slit complexes, and repulsion was mediated by Unc5-Netrin, Robo-Slit, and Neuropilin-Semaphorin complexes. Additionally, we learned that classical morphogens, such as Wnt and Shh, also function as chemoattractants. Most recently, we have found that several of these chemotropic factors, such as Netrin and Semaphorin, play parallel roles in guiding vascular growth. My current research aims to expose the extent of this parallel and apply this knowledge to design therapies promoting neural regeneration after injury, as well as inhibiting tumor angiogenesis.

Introduction

The guidance of neuronal and vascular growth is essential to proper development of an organism. The molecular signals responsible for this guidance were once, however, a mystery to the scientific community. My research over the last twenty years has transformed that century old textbook void into a wealth of knowledge.

Upon earning my doctorate in physiology at University College London in 1987, I returned to the U.S. to complete my postdoctoral fellowship at Columbia University in collaboration with Jane Dodd and Thomas Jessell, whom studied the molecular events regulating the early development of the mammalian central nervous system (CNS). Particularly, the embryonic mouse spinal cord provided an ideal model for assessing the growth of numerous populations of neurons extending their axons throughout the developing organism. Among these axon subtypes, we began studying the growth of commissural axons. These differentiated axons follow a stereotyped path from the dorsal spinal cord towards the ventral midline where they cross the commissure and extend their branches rostrally without ever re-crossing the commissure. Our initial research showed that the floor plate of the neural tube controls dorsoventral patterning of neuron types during embryonic development, including commissural neurons (1). More specifically, we found the floor plate secretes a diffusible factor that orients commissural axon growth *in vitro* by acting as a chemoattractant (2, 3).

In 1991, I set out to identify that chemoattractive factor. I made the move to University of California, San Francisco where I began teaching and researching as an investigator for the Howard Hughes Medical Institute at Stanford. There, in collaboration with close colleagues Corey Goodman and Thomas Kidd, we separated and identified the attractive and repulsive ligand-receptor complexes responsible for commissural axon guidance. Additionally, we characterized the crosstalk that occurs between these complexes on the growing axon, at different landmarks of their growth.

When I was offered my current position as Senior Vice President of Genentech in 2003, the focus of my research expanded to meet the biotherapeutic visions embodied by the company. Here, I have applied the discoveries of neuronal chemotropic factors to vascular tissue growth and shown them to play parallel roles between these tissues. Furthermore, in search for a treatment for neurodegenerative diseases and neural injury, I have investigated the controversial potential for CNS regeneration by manipulating expression of the neuronal growth inhibitory molecule, Nogo.

Neuronal and vascular tissue alike are blind to the fate of their growth. I have been privileged to discover some of the molecular factors guiding these fates. Almost certainly, many more remain undiscovered, and, with luck, future research will reveal them. Ultimately, knowledge of these factors may then guide treatment of relevant neural and vascular disorders, such as Alzheimer's disease and tumor angiogenesis. In this paper, I will tell the tale of commissural axon growth through our discovery of these chemotropic factors, show their parallels to vascular tissue growth, and express my outlook on CNS regeneration.

Commissural Axon Guidance

The Birth of Chemotropic Factors: Netrin

In 1994, after three years of publishing nothing but reviews on axon guidance, myself, Tim Kennedy and other colleagues purified, from embryonic chick brain extracts, the floor plate derived chemoattractant Drs. Jessel, Dodd, and I previously discovered. We christened them netrins,; the root "netr" originates from the Sanskrit language, and means "one who guides" (4). The netrins came in two isoforms, Netrin -1 and -2. They are homologous to the *Caenorhabditis elegans* protein UNC-6, which is similarly required for growth cone guidance and mesodermal cell migration in the developing nematode (4). Hybridization of the embryonic rat spinal cord with netrin-1 and -2 riboprobes confirmed that their expression occurs at the ventral midline floor plate (5). Moreover, the diffusion of netrin-1 in the developing rat spinal cord forms a protein gradient emanating from the floor plate (6). We also found that expression of diffusible recombinant netrin-1 and -2 by cells *in vitro* was sufficient to elicit commissural axon outgrowth from cultured rat dorsal spinal cord explants (4,5). To fortify evidence for netrin as a chemoattractant, we developed a homozygous netrin-1 knockout mouse line, in which the floor plate failed to cause commissural axon outgrowth (7). Still, we had no notion of

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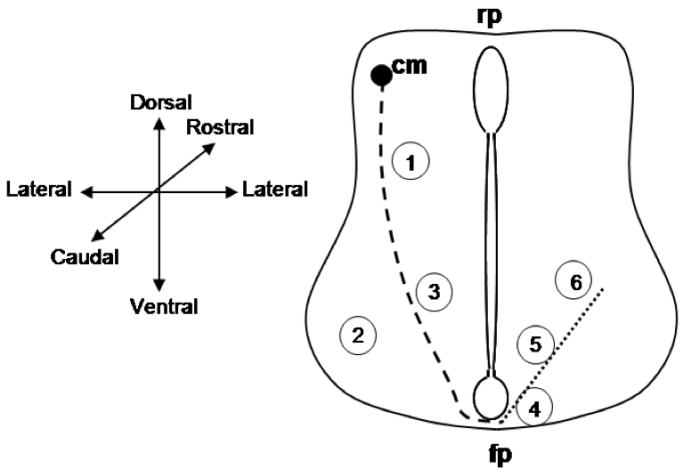

Figure 1. The Role of Chemotropic Factors in Guiding Commissural Axons. A rat model was used to track the path of commissural axon growth through the embryonic spinal cord and identify the influence of chemotropic factors at steps along this path. (1) Diffusible Netrin-1 and Shh gradient from fp directs cm growth from the dorsal sc towards the ventral commissure through respective cm DCC and Smo receptor expression. (2) Lateroventral expression of Unc5b netrin receptor on neurons outside cm path repels them from the commissure. (3) Rig-1 expression on pre-crossing cm prevents premature sensitivity to the Slit chemorepellant expressed ventrally. (4) Increased post-crossing cm expression of Robo-1 & -2 sensitizes axons to Slits chemorepellent activities, preventing axons from reentering the commissure. (5) Increased Neuropilin-2 expression on post-crossing cm sensitizes them to fp Semaphorin3 expression to repel axons from the commissure and guide them rostrally. (6) Wnt protein gradient guides cm growth rostrally by binding Frz receptor on cm (fp - floor plate, rp - roof plate, cm - commissural axon, sc – spinal cord).

how extracellular netrin exerted its chemoattractive effects on the neuron to direct growth.

Looking back to lessons from the elegant nematode, it was established that the *unc-5* and *unc-40* genes, encoding for transmembrane proteins, were implicated in UNC-6 dependent growth cone guidance (8). We found that the vertebrate protein Deleted in Colorectal Cancer (DCC) is a homolog of UNC-40 expressed specifically on developing commissural axons (8). We felt certain DCC was the Netrin receptor when cells expressing recombinant DCC bound netrin-1. Moreover, when we blocked DCC's extracellular domain using DCC blocking antibodies on dorsal spinal cord explants, a dose-dependent reduction in commissural axon outgrowth resulted (8). Thus, demonstrating that netrin mediates chemoattraction through DCC (Figure 1). We later showed that Netrin signal transduction through DCC depends on the cell-autonomous expression of heparan sulfate (HS), as evidenced by the commissural axon guidance deficits induced by Wnt-Cre driven axonal HS ablation in mice (9).

Still, we did not know what prevented other neuron populations from being drawn to the Netrin signal when DCC expression extended laterally from commissural axons to sensory and motor neuron columns as well (8). We hypothesized that the second *C. elegans* transmembrane protein, UNC-5, may hold some of the answers as it was implicated in *C. elegans* growth cone repulsion (10). I in collaboration with the Stein lab at Yale, discovered two rat homologues of UNC-5, UNC5H1, and UNC5H2 are expressed lateroventrally in the embryonic rat spinal cord (10). We also found both UNC5H1 and UNC5H2 show high binding affinities for Netrin, and their expression co-localized with DCC in embryonic lateroventral neuron populations (10). The simultaneous expression of DCC and UNC5 on these axons confounded the probability that Netrin mediated attraction and repulsion separately through DCC and UNC5 respectively. Thus, we hypothesized that UNC5 and DCC cytoplasmic domains interact to generate the repulsive effects of Netrin. We confirmed this hypothesis by producing chimeric forms of DCC and UNC5 receptors and blocking their cytoplasmic domains using specific antibodies (11). We found that an association between the UNC5 DCC binding (DB) domain and the DCC P1 domain mediate the conversion of Netrin attraction to repulsion (11; Figure 1). This helped explain the selective attraction of commissural axons to the ventral midline in spite of the diffuse Netrin gradient.

The Commissure: A Role for Robo and Slit

Now that we had a sense for how the commissural axons reached the ventral midline, the next obvious question became what signals them to cross and remain contralaterally. In a large scale *Drosophila* mutagenesis, the *roundabout* (Robo) gene was implicated in preserving the normal single projections of axons across the midline, by preventing their recrossing (12; Figure 1). Drs. Corey Goodman, Thomas Kidd and myself discovered that Robo was expressed most heavily on developing axon growth cones and filopodia in *Drosophila,* after they crossed the ventral midline (13). More importantly, we showed that Robo expression was conserved between fruit flies and mammals. Its transcripts were prevalent in the embryonic rat spinal cord and co-localized heavily with DCC expression (13). This suggested to us that an interaction occurs between the two receptors, but at the time, the ligand binding Robo remained unknown.

Within the next year, Dr.'s Goodman and Kidd identified the Robo receptor ligand in *Drosophila* as the Slit protein (14). I joined them to show evolutionary conservation of Slit among mammals (rats) in three isoforms, Slit-1, -2, and -3 (15). Slit and Robo transcript expression in the embryonic rat spinal cord overlapped heavily at the floor plate where commissural axons cross the ventral midline, as well as lateroventrally in the area of developing motor neuron columns (15). In support of our hypothesis that Slit acts as a chemorepellent through Robo, recombinant

expression of the Slit-2 isoform, which showed proteolytic processing, acted as a diffusible chemorepellent *in vitro* for developing motor axons (15). This explained motor axon repulsion as guided by both the Slit-Robo and Netrin-UNC5 complexes, but failed to address (a) why commissural axons are not repelled from the ventral midline when they are potentially subject to Slit-Robo repulsion pre-commissure crossing, (b) nor what causes them to lose responsiveness to floor plate Netrin expression post-crossing and repel them rostrally in the spinal cord.

It was only in my more recent research that I stumbled across an answer to the first question with colleagues at the Howard Hughes Medical Institute. We discovered a divergent member of the Robo family of receptors that we have named Rig-1. Like other Robos, it binds Slits, but unlike other Robos, it is expressed more heavily on the pre-crossing portion of commissural axons (16). We developed a Rig-1 null mutant mouse line to investigate its role in commissural axon guidance. Interestingly, these mutant commissural axons showed premature responsiveness to Slit, which prevented crossing at the midline – a phenotype partially rescued by either removal of Slit or Robo-1 expression (16). Thus, we concluded that Rig-1 prevents premature Slit sensitivity to facilitate commissural axon guidance by Netrin toward the ventral midline (Figure 1).

To understand why commissural axons lose responsiveness to Netrin chemoattraction post-crossing, the Stein lab and I looked for a potential interaction between DCC and Robo in developing spinal axons of *Xenopus*. We learned that Robo activation by Slit silences Netrin mediated attraction, while maintaining Netrin's neurotrophic effects (17). By developing chimeric Robo and DCC receptors with various cytoplasmic domain deletion constructs, we determined that this silencing occurs through direct binding of the Robo CC1 cytoplasmic domain to the DCC P3 domain (17).

Post-Crossing Repulstion: SemaphorinIII & Slit

To better understand guidance of commissural axons post-crossing, I must take a step backward to introduce chemotropic factors that play a major role in patterning sensory axon growth. Corey Goodman and I discovered the expression of the chemorepulsive ligand SemaphorinIII (SemaIII) in the mammalian ventral spinal cord. SemaIII patterns sensory projections of the dorsal root ganglia that exit the spinal cord dorsally via chemorepulsion without inhibiting the ventral growth of commissural axons (18). Through expression cloning for SemaIII binding proteins in the embryonic rat spinal cord, I discovered the transmembrane protein Neuropilin. Through Neuropilin, SemaIII induces chemorepulsion and growth cone collapse (19).

As commissural axons lose responsiveness to chemoattractant forces post-crossing, however, it follows that they may also gain responsiveness to chemorepellent forces. Such is the role of Slit binding to Robo (14,15). This led me to investigate whether commissural axons gain responsiveness to SemaIII post-crossing as well. I found that post-crossing, but not pre-crossing axons were inhibited by recombinant expression of both SemaIII and Slit-2 (20). This indicated that expression of SemaIII by midline and nonmidline tissue functions in conjunction with Slit to prevent commissural axons from re-entering the midline post-crossing (Figure 1).

Morphogens Are Chemotropic Factors Too

Classical morphogens, such as Sonic Hedgehog (Shh) and Wnt, are molecules expressed in a developing organism that serve to pattern tissue differentiation according to their concentration along a gradient. Chemotropic molecules function in a similar fashion via diffusion to pattern axon growth. My previous research had shown that in the absence of Netrin or DCC, commissural axons generally failed to reach the ventral midline; still, a small population does (7). This suggested that other factors aid Netrin to guide their growth. Shh was a likely suspect in this case given its secretion by the floor plate. In collaboration with Elke Stein and Frederic Charron, I found that recombinant Shh expression *in vitro* caused commissural axon turning and that blocking its receptor, Smoothened, among Netrin-/- mice *in vivo* eliminated all attractant activity of the floor plate (21; Figure 1). Complementarily, my old postdoctoral colleague Jane Dodd showed that Bone Morphogenic Proteins (BMPs) expressed by the roof plate function to repel commissural axons from the dorsal spinal cord (22). Morphogens also hold the key to why commissural axons turn rostrally after crossing the midline. I implicated a rostrally increasing Wnt gradient as a chemoattractive force guiding commissural axon growth towards the brain through the Frizzled receptor (23; Figure 1). The dual chemotropic and morphogenic roles of these molecules exemplify the functional conservation prevalent in all aspects of neuronal guidance. In this model a single molecule serves multiple functions according to its spatiotemporal expression during development.

Parallel Systems: Guiding Vascularization

Functional conservation provided the basis for my hypothesis that vascular growth could be guided by the same molecules as neuronal growth. In 2003, I made the move from my position as a Primary Investigator in academia to fill the administrative role as a Senior Vice President for the industrial biotech giant Genentech. This meant adapting and expanding my research focus to meet the therapeutic aims of the company.

One of the foremost goals of Genentech is to identify therapeutic targets for cancer treatment. The growth of any tumor depends on the growth of new blood vessels into the tumor to provide a source of raw cellular materials and nutrition, a process termed angiogenesis. This growth of new vascular tissue suggested to me that chemotropic signaling molecules would be required to guide such growth. Throughout the human body, vascular and neuronal tissue form networks that run in parallel. More interestingly, they produce signals that mutually guide each other's development (24). Nerves produce vascular endothelial growth factor to guide vascular growth, while endothelial cells often produce neurotrophin-3 to attract axon outgrowth alongside the developing vasculature (24). As such, I have guided research at Genentech towards determining whether the chemotropic factors I helped to discover play similar roles in vascular morphogenesis.

Unlike axons, which represent an extension of a single cell, vascular tissue is composed of many endothelial cells held together by cell adhesion molecules, such as integrins, expressed on their membranes. Thus, interruption of these cell-cell adhesions is critical for developing new vasculature. We have recently discovered that SemaIII plays a critical role in facilitating angiogenic plasticity by antagonizing integrin-mediated adhesion (25). Moreover, we found that these effects are transduced through the Neuropilin (Nrp) receptor, as in axon guidance (26). Although in addition to binding SemaIII, Nrp also binds VEGF on endothelial cells. To determine the role of Nrp in vascularization, we applied Nrp blocking antibodies to mouse retina and tumor models of vascular development (26). We found that blocking Nrp significantly reduces vascular density in both tissue types (26). Thus, SemaIII does not act as a chemorepellent to endothelial cells as it

does to neurons. Instead of inhibiting vascular growth, it seems to promote it by reducing cell-cell adhesion to facilitate reorganization of pre-existing vasculature (Figure 2).

In contrast to SemaIII, we found that the chemorepellent quality of Netrin signal transduction through UNC5b, an isoform of UNC5, was actually conserved (27, 28, 29). Among mice and chicks, UNC5B expression was localized to arterial endothelial cells and sprouting angiogenic capillaries (28,29). In zebrafish, we learned that Netrin binding to UNC5B on endothelial filopodia causes filopodial retraction (27). Disruption of this interaction by knocking out UNC5B induced excessive angiogenesis (27). We further supported this Netrin-UNC5B mediated inhibition of sprouting angiogenesis among developing mouse and chick embryos (28, 29; Figure 2). All of these experiments point to UNC5B as a prospective anti-angiogenic target to prevent tumor growth. Moreover, they support the need for further research into the conserved role of chemotropic factors among vascular and neuronal networks.

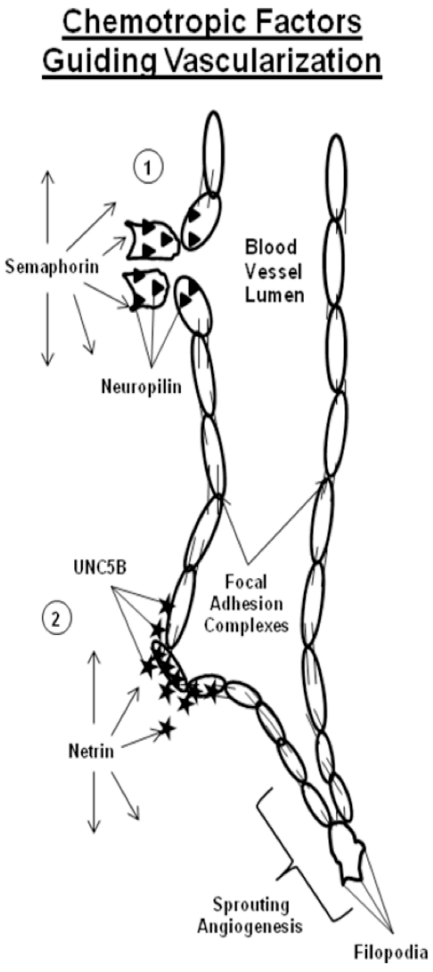

Figure 2. The Role of Chemotropic Factors in Guiding Vascularization. This model portrays the impact of SemaIII-Nrp and Netrin-UNC5B ligand-receptor complexes on vascular growth in a developing blood vessel. (1) SemaIII signaling through Nrp on endothelial cells interrupts cell-cell focal adhesion complexes to facilitate cell mobility that is necessary for vascular plasticity and outgrowth. (2) Netrin signaling through UNC5B repels endothelial filopodia to inhibit new vascular growth, termed sprouting angiogenesis.

CNS Regeneration: Nogo and…Netrin?

The most attractive, yet most elusive target for biotherapy is the mammalian central nervous system (CNS) due to the numerous innate roadblocks to regeneration present. Among these barriers are the numerous neuronal growth inhibitory molecules found in CNS myelin. Within the last five years, a subsection of my research has been dedicated toward investigating the role of the potent, CNS specific growth inhibitor Nogo in regeneration (30). Previous research showed that antibody blocking of Nogo function could stimulate CNS axonal recovery post-injury (31).

My lab developed Nogo-A/B and Nogo-A/B/C knockout mice and performed spinal cord slice injuries on these mice to test their capacity for regeneration. My lab performed these experiments at the same time as the labs of my colleagues, Drs. M. Schwab and S. Strittmatter. Their Nogo knockout mice respectively showed either partial or complete corticospinal tract regeneration, while, at odds with their results, my Nogo knockout mice showed absolutely none (31, 32, 33). Distressed by these dissonant results, Dr. Strittmatter even graciously offered to have the member of his lab who performed the injuries do the same for ours. However, this still yielded the same results (31). I fortified the validity of these findings in a later study via genetic deletion of the Nogo receptor *in vitro* and via an *in vivo* mouse spinal cord injury model (34). In both cases, preventing Nogo's growth inhibitory axons among injured neurons was ineffective at promoting regeneration (34). My lab's recent research suggests that blocking Nogo function only induces acute growth cone collapse on an axon post-injury, while chronic growth inhibition could be attributed to other myelin inhibitors like Oligodendrocyte Myelin glycoprotein (OMgp) (35). This suggested that perhaps a more cumulative therapy targeting myelin growth inhibitors may show more promise to promote CNS regeneration.

While my lab found that Nogo did not promote regeneration, it did show its promise in delaying the progression of neurodegeneration. In a mouse model of multiple sclerosis, Nogo-A vaccination generated an immune response that blunted behavioral signs of the disease, as well as reduced demyelination and axon damage associated with its progression (36). This points to a plausible role for targeting Nogo to treat the wide spectrum of neurodegenerative disorders prevalent in the ever-aging population.

Within the last two years, my research into regeneration has led me to revisit the role of Netrin. I initially implicated Netrin as a repulsive cue that limits CNS remodeling post-injury. In mice, it plays this role by repelling adult neural stem cells away from the floor plate post-injury (37). However, even more recently, my lab found that Netrin plays a more broader role in preventing regeneration as a novel myelin based growth inhibitor, which transduces its inhibitory signal through UNC5 on spinal motor neurons (38). This only compounds the puzzle of regeneration by implying that other chemotropic factors may also function to promote or inhibit CNS regenerative growth.

Conclusions

Our discoveries of chemotropic receptor ligand complexes has allowed us to map an elementary path to commissural axon guidance (Figure 1). While we have implicated these molecules in axon guidance, we know so little of the downstream mechanisms transducing their signals. Nor have we investigated any potential differences in their functioning between the central and peripheral nervous systems. More relevant to our current research, we do not

know to what extent these molecules play parallel roles to pattern vascular growth.

Nonetheless, we have discovered that several of these molecules are functionally conserved in vascular growth (Figure 2). More importantly, knowledge of their function will help us develop new biotherapies to treat vascular diseases, such as the angiogenic process associated with tumor development. Additionally, our recent research into the potential for CNS regeneration, yet again, points to these chemotropic factors as therapeutic targets in cases of injury as well as neurodegenerative disease.

Much like Tiresias of *Antigone*, neurons and endothelial cells alike are blind to the fate of their growth. They require guidance, but they guide us too. The wisdom we have gained through learning of their growth has given birth to a new paradigm in development for how we explain cell migration and tissue patterning.

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