

# Is Substrate Stiffness an Axon Guidance Cue?

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## Introduction

During the development of the brain, each of a trillion neurons makes thousands of specific synaptic connections. <sup>1</sup> They extend axons which travel up to several feet, navigating around a range of obstacles to hone in on their final target. <sup>2</sup> For decades, researchers have been trying to uncover the mechanisms by which neurons extend their axons with such remarkable precision. Over a century ago, Ramon y Cajal proposed that axon growth might be guided by long-range chemical cues. <sup>3</sup> Today, there is substantial evidence that axon guidance is mediated by a complex combination of chemical and contact guidance signals. A deeper understanding of this process may facilitate improving methods for regeneration of damaged axons as well as prevention of nervous system wiring defects that contribute to dyslexia, cerebral palsy, and mental retardation. <sup>2</sup> In this paper, I propose an experiment to investigate whether differences in substrate stiffness guide axon extension.

## Growth Cones

An advancing neurite is tipped at its leading edge by a growth cone, a structure with actin-rich lamellipodia and filopodia that develop in the direction of movement. (See Fig. 1) Neurite outgrowth is cyclic, meaning that growth cones exhibit search, displacement and rest phases, instead of maintaining a constant pace. It appears that a growth cone extends filopodia to probe the environment, and when a guidance cue is detected it moves in the appropriate direction by exerting a traction force on the substrate and pulling on the neurite to extend it. <sup>4,5</sup>

## Guidance Cues

There are four main types of guidance cues:

- 1) Chemoattraction
- 2) Chemorepulsion
- 3) Contact attraction
- 4) Contact repulsion

Chemical cues are provided by secreted diffusible molecules like semaphorins and netrins that act over a long range. Contact cues are short-range and arise from interactions between non-diffusible cell surface and extracellular matrix (ECM) molecules. The long range cues act to push and pull the axon from a distance while the short range cues hem the axon into a more specific path. <sup>3</sup> (See Fig. 2) Another type of guidance behavior is selective fasciculation, where new axons “choose” to grow along pre-existing axons (these paths are also known as fascicles) switching from one path to another at specific points. <sup>3</sup>

However, it is not always possible to fit each guidance cue neatly into these categories. For example, diffused molecules may bind to the cell surface or the ECM, and alter the short-range contact cues. Furthermore, a growth cone may change its state and respond differently to the same guidance molecule, i.e. an attractant may become repellant, or vice versa.<sup>1</sup> This property is useful because a growth cone typically navigates to several intermediate targets before reaching its final destination, and it must modulate its responsiveness in order to find each successive target.<sup>3,6</sup> Furthermore, there is a great deal of redundancy amongst the guidance cues. Usually, removing any single mechanism will not have a significant effect. Although this property makes the guidance system very robust, it also increases the difficulty of experimentally determining and characterizing guidance cues.<sup>3</sup>

### **Durotaxis**

While a great deal of attention has been paid to identifying and characterizing molecular guidance cues, little work has gone into assessing the influence of physical properties of the substrate on growth cone motility. It is known that the movement of other types of migratory cells can be affected by purely physical interactions. For example, fibroblasts<sup>7</sup> and vascular smooth muscle cells<sup>8</sup> have been shown to move preferentially towards stiffer substrates, a process known as durotaxis. Lo, Wang, Dembo, and Wang found that a fibroblast will avoid moving onto a softer substrate, as well as turn around and move toward the increased tension created by a microneedle “tugging” on the gel.<sup>7</sup> In a similar study, Wong, Velasco, Rajagopalan, and Pham demonstrated that vascular smooth muscle cells cultured on a substrate with a gradient of stiffness will move towards stiffer regions.<sup>8</sup>

The effect of substrate stiffness on axon guidance has not been extensively studied. Flanagan, Ju, Marg, Osterfield and Janmey demonstrated that neurites have a lower extension rate and fewer branches on stiffer substrates. Moreover, neurites appear to be much more sensitive than fibroblasts to substrate stiffness. Their morphology changed on substrates with a stiffness range of 500-5500 dyne/cm<sup>2</sup>, while fibroblasts react to the 140,000 – 300,000 dyne/cm<sup>2</sup> range.<sup>9</sup>

Although the work of Flanagan et al. makes it clear that neurites can be physically affected by substrate deformability, it does not completely answer the question of whether substrate stiffness plays a role in axon guidance. It is possible that one mechanism by which growth cones sense nearby axons or intermediate targets is by detecting changes in ECM tension. This mechanism would not be apparent in an experiment that uses gels with a uniform stiffness. It makes sense that substrate stiffness affects how quickly neurites grow (as demonstrated by Flanagan et al.), but do variations in stiffness change the direction of growth cone movement?

### **Experiment**

My experiment proposal is designed to better understand how the direction of neurite outgrowth is affected by substrate stiffness. It is based on the gradient experiment designed by Wong et al. to study vascular smooth muscle cells.<sup>8</sup> Hopefully, it will better determine how growth cones react to a substrate with a gradual change in stiffness.

Here are the basic steps of the experiment:

- 1) Create polyacrylamide gels. Regulate their stiffness by controlling the intensity of UV light during their photopolymerization with mask patterns laser printed on transparencies. The first set of gels will use filters with a uniform darkness, but each gel will have a different shade of filter. These will serve as the control gels, with uniform stiffness. The second set will have a gradient of stiffness across the gel. (See Fig. 3) All gels will be coated with Matrigel to provide a constant concentration of ECM proteins appropriate for neurite extension.
- 2) Measure the stiffness of each gel at several points. The controls created with uniform filters should have a uniform stiffness across the gel, and the stiffness across the gradient-filter gels should increase as the gradient becomes lighter. The filters and gel creation process will need to be modified if this is not the case.
- 3) Culture neurons on each gel. Take a time-lapse movie. Extrapolate growth cone velocity, number of branches, and general morphology information from the movie.
- 4) Repeat with multiple trials to insure consistency across the results.

### **Expected Results**

In the control gels, I expect to see results similar to Flanagan et al., that is, neurons cultured on stiffer gels will have decreased extension speeds and less branching. On the graduated gels, however, I can think of three different possible results which are sketched in Fig. 4. The first possibility (A) is that stiffness has no effect on the direction of growth cone migration. Instead, the axons will grow in random directions and their lengths will be determined by the stiffness. On the stiffer side, neurites will be shorter and sparser, while the softer side will have longer neurites with more branches. This result indicates that substrate stiffness gradients are not a guidance cue. The second possibility (B) is that growth cones that begin to on the stiffer side will turn towards the softer side, creating neurites that all flow in a similar direction. The third possibility (C), combining the work of Flanagan et al. and Wong et al. is that the growth cones will be attracted to the stiffer side, but the axons will be slower and less branched. Results (B) and (C) indicate that substrate stiffness gradients could be a guidance cue.

Although (C) combines the results of two previous studies, I think it is the least likely scenario. If the neurites are slower to extend in stiffer substrate but move toward stiffer substrate, over a certain time period neuron growth will be stunted compared to the other possibilities. If growth cones travel faster in softer substrates, it does not make sense that they would have a preference for stiffer substrates like fibroblasts and smooth vascular muscle cells do. Moreover, it has already been demonstrated that fibroblasts and growth cones react to different ranges of stiffness, so it is not unlikely that their behaviors may be opposite. (Fibroblasts move toward stiffness, growth cones may move away from it.) Thus, I predict that growth cones will have some significant response to substrate stiffness, but they will behave differently than fibroblasts or muscle cells. Perhaps there is even an optimal range of stiffness that attracts growth cones. I definitely expect to see results in the 500-

5500 dyne/cm<sup>2</sup> range where Flanagan et al. found changes in neurite morphology, but it is also possible that the direction of growth cone movement is responsive to values outside of this range.

### **Advantages**

The experimental set up is very simple and straightforward. It does not require special out-of-the ordinary equipment, and the results should be easy to quantize and interpret.

### **Limitations**

Since the state of a growth cone can determine its reaction to a guidance cue (attract, repel, or neutral) it is possible that the results of this experiment will only be representative of growth cone behavior in a certain state. Perhaps growth cones exhibit a stronger/weaker reaction to substrate stiffness when they are triggered by another guidance cue. This type of information will be hard to determine from this experiment alone. Furthermore, it is likely there will be inconsistencies in the gels that will lead to experimental discrepancies. Finally, these experiments are performed on 2D gels *in vitro*, which may have characteristics that are not consistent with how growth cones react *in vivo*.

### **Experimental Variations**

Depending on the results from the first part of the study, there are several other experimental paths that could be followed. If the growth cones respond to changes in stiffness, I could also tug on the gel with a microneedle (like Wong et al. did) to see if the growth cone's path can be manipulated. Furthermore, it would be intriguing to combine a stiffness gradient with a chemical gradient and observe which has more power over the response of the growth cones.

### **Conclusion**

Axon guidance is a complex process with numerous interplaying factors. Understanding how substrate stiffness affects growth cone movement will fit another piece into the puzzle. This experiment may help to better explain how growth cones sense their targets, and it may even open doors to new therapies that direct axon growth to restore damaged nerves. At the very least, it will contribute to the growing body of knowledge of how substrate properties affect cell movement.

## Figures

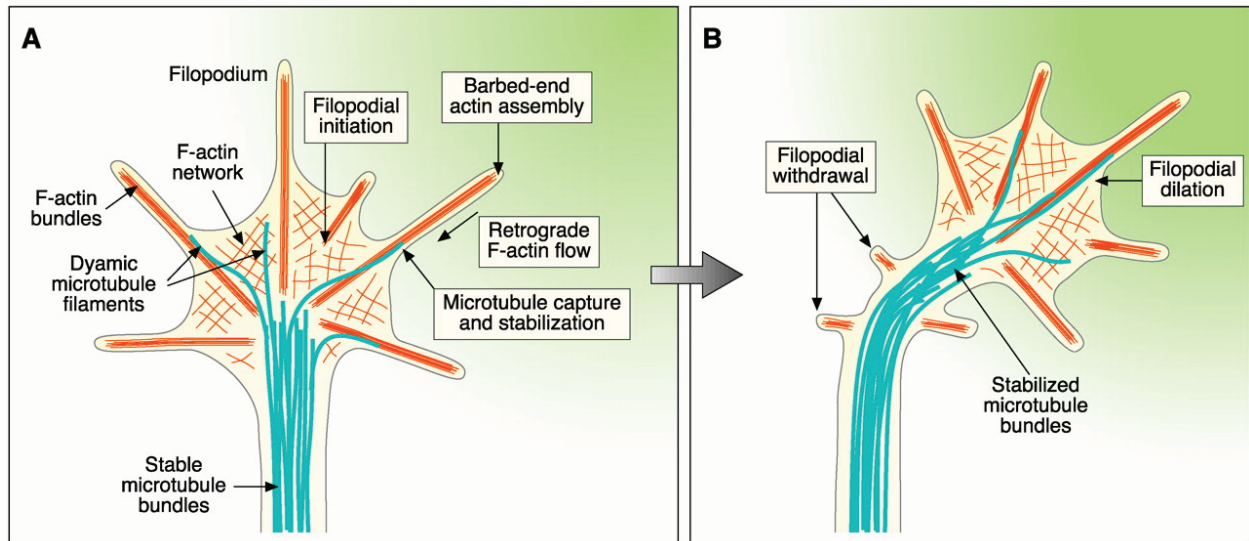


Fig. 1: Response of a growth cone to a chemical attractant (green).<sup>6</sup>

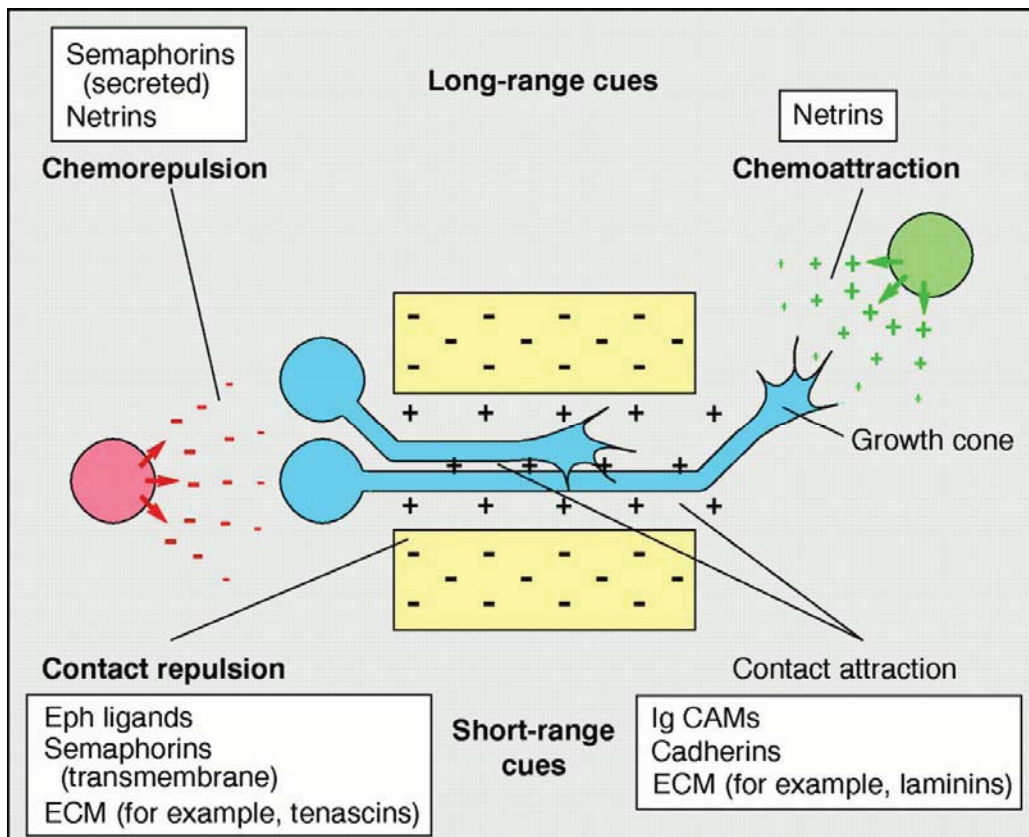


Fig. 2: Guidance cues. A growth cone is pushed from behind by long-range chemorepellent (red), pulled by a chemoattractant (green) and hemmed in by short-range attractive (gray) and repellent (yellow) cues.<sup>3</sup>



Fig. 3: Laser-printed filters for gels. The first six on the left will create control gels with a uniform stiffness. The last filter on the right is for creating the experimental gel with a stiffness gradient.

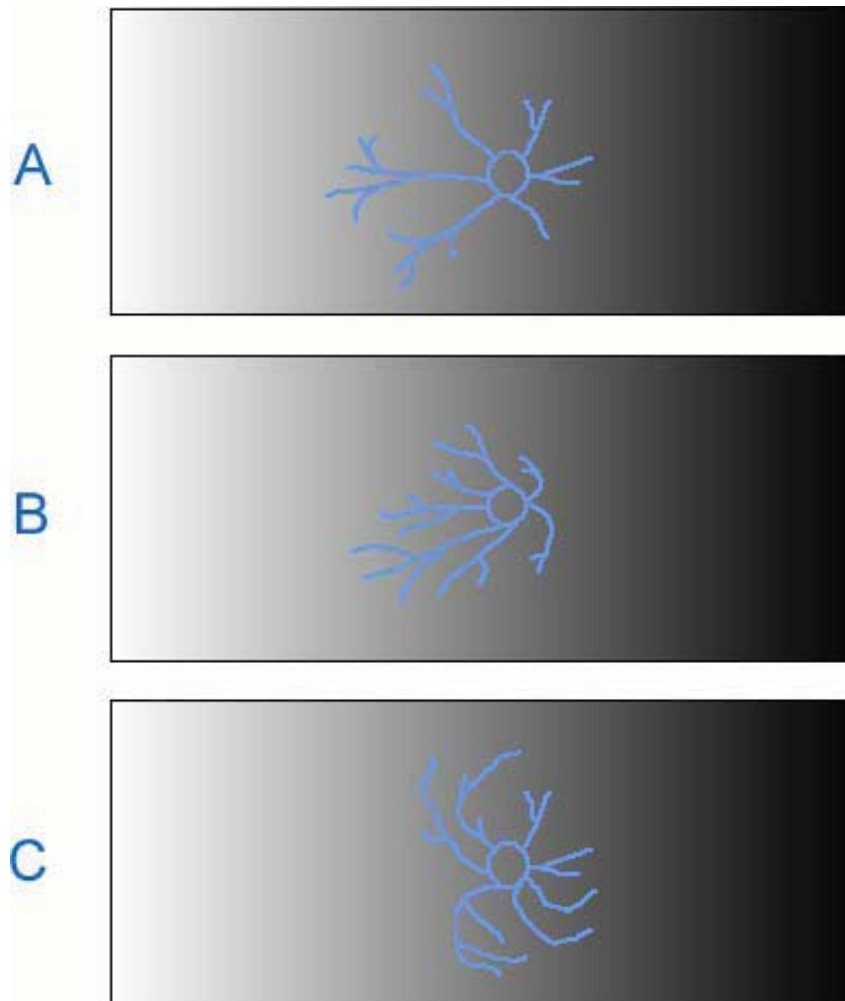


Fig. 4: Possible experimental results. A: Stiffness has no effect on the direction of growth cone movement. B: Growth cones are attracted to softer substrates. C. Growth cones are attracted to stiffer substrates, like fibroblasts and vascular smooth muscle cells are.



## References

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