

The effects of laminin substrate on axon pathfinding in living sympathetic neurons

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Introduction

Neuron formation involves the extension of processes from the cell body. These processes consist of dendrites and axons of the neuron which are necessary for signal reception and transduction, respectively. When combined, individual neurons form complex networks, and for proper formation of these networks, the axon of one neuron must correctly interact with the dendrites of another (Kandel, et al., 2012). Axon pathfinding is a current topic of study that is not well understood. It is understood, however, that axon pathfinding is guided in part by genetics and in part by the environment in the form of at least four different biochemical mechanisms—contact attraction, chemoattraction, contact repulsion, and chemorepulsion (Tessier-Lavigne and Goodman, 1996). Other processes of axonal pathfinding include the binding of cell adhesion molecules (Chiba and Keshishian, 1996) and availability of ATP (Morris and Hollenbeck, 1993). *In vivo*, the pathways for neuron growth are often laid out by bodily tissues and specific proteins creating a local environment optimal for the specific neuron type (Clark, Britland and Connolly, 1993). *In vitro*, these local environments can be mimicked by the use of different substrates in varying concentrations.

The substrate laminin is made up of laminin proteins that are found in the basal lamina and help make up the extracellular matrix throughout the body; they are important in their effects on cell survival, differentiation, migration and adhesion to different surfaces (“Laminin,” 2013 and Timpl, et al., 1979). Patterns of laminin can be altered *in vitro* and can act as different guidance cues changing branching configurations of neurons (Clark, Britland and Connolly, 1993). Neuron growth is affected because of the contact attraction of binding sites in the laminin protein. *In vivo* it has been shown that the interaction between nidogen-binding sites in the gamma subunit of laminin integrin proteins on the neuronal cell surface is necessary for axon pathfinding and growth cone direction (Bonner and O’Connor, 2001).

The adhesive property of laminin proteins allows for the binding of filopodia extensions coming from the neuron cell body. In the primary stages of neuron growth, axons and dendrites have not yet differentiated. Both begin as filopodia, or thin, cytoplasmic spikes containing actin filaments (Small, et al., 2002). Filopodia exhibit highly dynamic motion extending and retracting depending on mechanical cues such as binding capabilities to the surfaces on which they lay (Flanagan, et al., 2002). When binding occurs, actin filaments of the filopodia depolymerize creating a gradient

that leads to a rush of cytoplasmic fluid to the area forming a web-like structure called lamellipodia. This process repeats itself to form a growth cone which continues extending outward from the cell body by the guidance of extracellular cues. The resulting axon becomes engorged with organelles and proteins specific to axon functioning, and the axonal pathway is formed (Dent and Gertler, 2003).

Studies performed *in vitro* have shown that axonal growth is correlated with laminin distribution (ie. Bonner and O'Connor, 2001, and Dodla and Bellamkonda, 2006). This study tested the initiation of axon pathfinding with *Gallus gallus* neurons plated on laminin and non-laminin surfaces. I hypothesized that due to the adhesive properties of laminin, neurons on active laminin substrate will have longer axons and shorter filopodia than those of neurons not on laminin. This could suggest that neurons on laminin surfaces will form axons more quickly than neurons not on laminin due to filopodia being more likely to bind to the surface, resulting in quicker formation of a growth cone. This finding could help demonstrate the importance of the role of laminin in forming neural networks.

Materials and Methods

Sympathetic Nerve Chains and Dorsal Root Ganglia were dissected from *Gallus gallus* embryos, dissociated and cultured by Morris's "Neurobiology Bio324- Primary Culture of Chick Embryonic Peripheral Neurons 1: Dissection" protocol (2013). However, DMEM was substituted for HBSS, and coverslips were treated in two different manners. All coverslips were treated with polylysine and two separate drops of laminin which was left to dry creating two circular layers of dried, denatured laminin. On half of the coverslips, a second drop of laminin was placed on top of the dried laminin circles to create the experimental condition of active laminin. The other half of the coverslips served as the control of dried laminin to ensure results were due to the active laminin proteins and not the extra thickness of the dried laminin surface.

A chip chamber was formed and the live cells were observed using Morris's "Neurobiology Bio324- Primary Culture of Chick Embryonic Peripheral Neurons 2: Observation of Live Unlabeled Cells" protocol (2013). Cells were observed at the Imaging Center for Undergraduate Collaboration at Wheaton College, Norton, MA with a Nikon Eclipse E200 microscope, which was aligned for Koehler illumination at 40x magnification under phase optics. Images of neurons on active laminin, off laminin, and on denatured laminin were captured using a Sony DFW-X700 Camera with a C-mount magnification of 1.0x and viewed on an Apple Mac G4 using the program BTV Version 6.0b1. Collaborators Ali McCarthy and I-Wen Lin also captured images used for this experiment (McCarthy, 2013 and Lin, 2013). Using the segmented line tool on program Image J Version 1.40g, lengths of filopodia extending from the cell body were measured in number of pixels and an average length was calculated for each neuron. Cell's axon lengths

were also measured. Extensions were determined to be axons if they measured more than 7 pixels wide 25 pixels from the cell body. This was because previous studies showed average axon lengths to be approximately 1 μm (ie. Chiu, 1987). Calculations were performed to find the number of pixels per micron, which was determined to be approximately 6.34 pixels/ μm ; hence 7 pixels appeared to be a good measure differentiating axons and filopodia. The distance of 25 pixels from the cell body was specified because lamellipodia could sometimes appear to be extensions greater than 7 pixels in diameter; however, they did not extend out 25 pixels from the cell body. If more than one extension met these conditions, the longest one was chosen, and if branching occurred in filopodia or axons the longest branch was measured.

Results

There was a great deal of diversity amongst the appearance of the cells. Some cells had developed relatively long axons and had few filopodia such as the neuron in Figure 1. Others had not yet developed axons, but had many filopodia extending from the cell body such as the neuron in Figure 2.

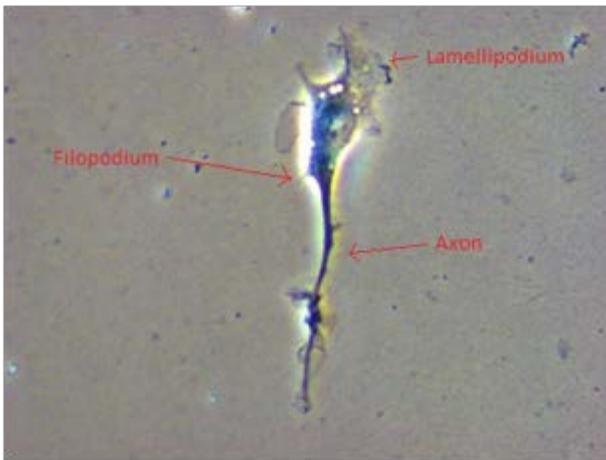


Figure 1- An example of a neuron found on the laminin substrate viewed at 40x magnification. The neuron contains one large axon as well as some smaller filopodia extending from the cell body. A lamellipodium can also be seen off of the top right portion of the cell body.



Figure 2- An example of a neuron found off the laminin substrate viewed at 40x magnification. The neuron does not contain an axon; however, many filopodia can be seen extending outward from the cell body.

Figure 3 below shows that the average length of filopodia is longer off laminin and shorter on active laminin and dried laminin when compared to average axon length. The numbers of cells that were able to be observed on each substrate type varied with an n-value of 11, 5, and 2 for off laminin, active laminin and dried laminin, respectively. Figure 4 displays the axon length versus the average filopodia length for each cell that was observed. A trend line for all data points shows axon length decreasing as average filopodia length increases.

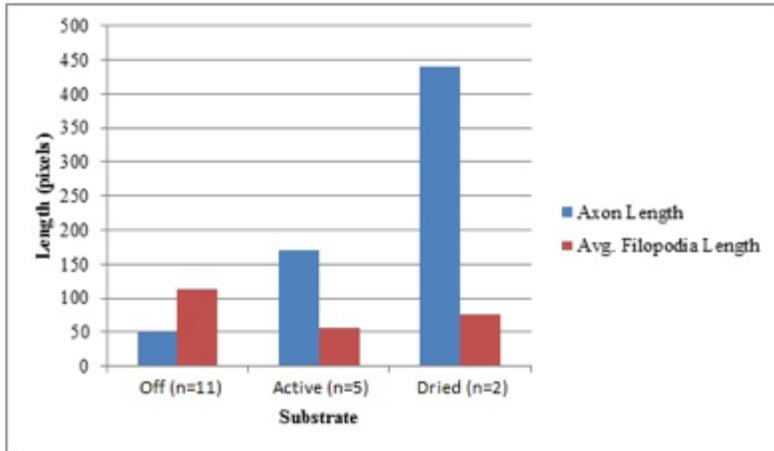


Figure 3- The average lengths of filopodia and axons in all observed neurons. Average filopodia length was longer than axon length off laminin, while the opposite trend occurred for neurons on active and dried laminin.

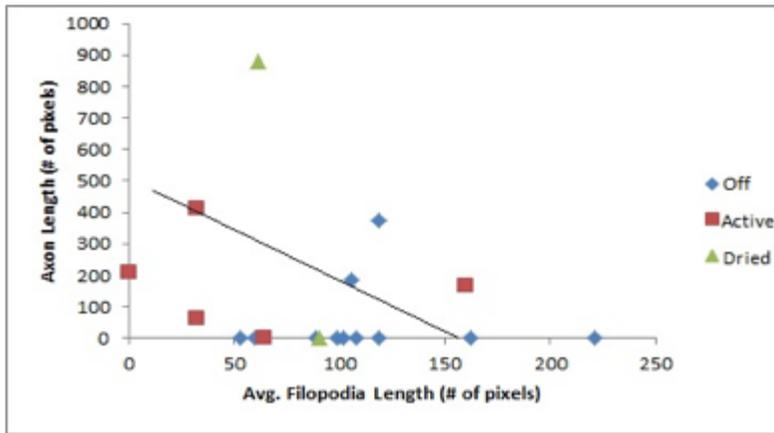


Figure 4- The average length of all filopodia on each neuron observed versus the axon length. Cells found off laminin (Off), on active laminin (Active), and on dried laminin (Dried) were observed. All lengths measured in pixels; 1 um contains approximately 6.34 pixels. The trend line for all data points shows that as average filopodia length decreases, axon length increases.

Discussion

The results support the hypothesis that neurons on laminin substrate will have longer axons and shorter filopodia than those on non-laminin substrate (Figure 3). Neurons on dried laminin also exhibited longer axons and shorter filopodia, when it was expected that the dried laminin neurons would share similar traits to those of neurons off laminin. This, however, could be explained by the small sample size of neurons on dry laminin (n=2). If this finding was seen repeatedly, however, it could be assumed that the contact attraction of laminin proteins is not the cause of faster

developing axons. Further experimentation would be needed to explain this finding.

Formation of an axon path is determined in part by contact-attraction molecules such as laminin (Tessier-Lavigne and Goodman, 1996). In order for axons to grow, extending filopodia must be able to bind to their surrounding surface. When there are fewer contact attraction molecules this binding is less likely to occur, and the filopodia must continue retracting and re-extending to form an axon path (Dent and Gertler, 2003). *In vivo*, it has been observed that laminin is essential to growth cone pathfinding (Bonner and O'Connor, 2001), so it is possible that *in vitro* the initiation of axon growth would take longer in the absence of laminin.

To show further support of the hypothesis that axon length will be greater and filopodia lengths will be lesser on laminin than off laminin, future studies could involve looking at time-lapse photographs to see the extension and retraction of filopodia and possibly axon formation. Another experiment could also be performed in which the cells are observed at different periods of growth. Since all cells in this experiment were plated and observed at the same time, it could be assumed that they had each experienced the same amount of growth time. If observations had taken place after longer growth times more cells could have developed axons and the lengths of those could have been compared. Another interesting study would involve looking at neurons on laminin/non-laminin boundaries, and determining the angle at which the axon grows. This could show a clear direction being “chosen” by the extending filopodia for axon growth.

References

- Bonner, J., & O'Connor, T. P. (2001). The permissive cue laminin is essential for growth cone turning in vivo. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, 21(24), 9782–9791.
- Buettner, H. M., & Pittman, R. N. (1991). Quantitative effects of laminin concentration on neurite outgrowth in vitro. *Developmental Biology*, 145(2), 266–276.
- Chiba, A., & Keshishian, H. (1996). Neuronal Pathfinding and Recognition: Roles of Cell Adhesion Molecules. *Developmental Biology*, 180(2), 424–432.
- Chiu, S. Y. (1987). Sodium currents in axon-associated Schwann cells from adult rabbits. *The Journal of physiology*, 386, 181–203.
- Clark, P., Britland, S., & Connolly, P. (1993). Growth cone guidance and neuron morphology on micropatterned laminin surfaces. *Journal of cell science*, 105 (Pt 1), 203–212.
- Dent, E. W., & Gertler, F. B. (2003). Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance. *Neuron*, 40(2), 209–227.

- Dodla, M. C., & Bellamkonda, R. V. (2006). Anisotropic scaffolds facilitate enhanced neurite extension in vitro. *Journal of Biomedical Materials Research Part A*, 78A(2), 213–221.
- Flanagan, L. A., Ju, Y.-E., Marg, B., Osterfield, M., & Janmey, P. A. (2002). Neurite branching on deformable substrates. *Neuroreport*, 13(18), 2411–2415.
- Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S. A., & Hudspeth, A. J. (Eds.). (2012). *Principles of Neural Science, Fifth Edition (Principles of Neural Science (5th ed.))*. McGraw-Hill Professional.
- “Laminin.” (2013, February 26). In *Wikipedia, the Free Encyclopedia*. Retrieved from <http://en.wikipedia.org/w/index.php?title=Laminin&oldid=540524499>
- Lin, I-Wen. (2013). Personal Communication.
- McCarthy, Ali. (2013). Personal Communication.
- Morris, R. L. (2013). “Primary culture of chick embryonic peripheral neurons 1: Dissection.” *Neurobiology Bio324 Notebook*.
- Morris, R. L. (2013). “Primary culture of chick embryonic peripheral neurons 2: Observation of live unlabeled cells.” *Neurobiology Bio324 Notebook*.
- Saleh, C. N., & Ehrlich, D. (1984). Composition of the supraoptic decussation of the chick (*Gallus gallus*). A possible factor limiting interhemispheric transfer of visual information. *Cell and tissue research*, 236(3), 601–609.
- Small, J. V., Stradal, T., Vignal, E., & Rottner, K. (2002). The lamellipodium: where motility begins. *Trends in cell biology*, 12(3), 112–120.
- Tessier-Lavigne, M., & Goodman, C. S. (1996). The molecular biology of axon guidance. *Science (New York, N.Y.)*, 274(5290), 1123–1133.
- Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., & Martin, G. R. (1979). Laminin--a glycoprotein from basement membranes. *Journal of Biological Chemistry*, 254(19), 9933–9937.