The permissive extracellular matrix cue laminin and its promoting effect on neurite outgrowth

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Introduction

The activity of neurite growth is influenced by the guidance cues in the local environment. Nerve processes are capable of detecting these cues and responding to them in order to determine which direction to extend for neurite outgrowth (Raper and Manson, 2010). During embryonic development, the nerve cells send out sensory protrusions to assess the environment (Tessier-Lavigne and Goodman, 1996). The guidance components in the surroundings provide the information for neurite outgrowth. After the cell integrates the guidance cues, neuritic outgrowth is then developed by the formation of axon and dendrite growth cones (Raper and Manson, 2010). According to Tessier-Lavigne and Goodman (1996), there are various classes of guidance cues: short-distanced, long-distanced, inhibitory, and attractive ones. Each guidance molecule signals and regulates the axon pathfinding's activity differently. As a result, depending on which specific cue is presented, the growth cone may be simulated, paused, or inhibited (Raper and Manson, 2010).

In order for neurons to perform neuritic regrowth in vitro (literally "in glass"), two components are required – the nutrition for nerve cells to survive and neurite-promoting substances (Manthorpe et al., 1983). These neurite-promoting substances are categorized into two classes: cell adhesion molecules (CAMs) and extracellular matrix (ECM) components (Clark and Connolly, 1993). ECM components are the molecules that abundantly exist in the extracellular matrix while CAMs are proteins on the cell membrane that bind with other cells or ECM molecules (Tessier-Lavigne and Goodman, 1996). Laminin is a large, cross-shaped ECM protein that promotes growth cone development and neurite pathfinding. Previous researches suggest that laminin is a permissive cue that provides the essential environment for neurons to grow and extend neurites on substratum (Bonnor and O'Connor, 2001). It has significant roles on neuronal adhesion, enhancing neuritic outgrowth, and orienting direction of nerve processes, and growth cone guidance (Dertinger et al., 2002; Hammarback et al., 1985; Letourneau, 1975). When presenting ECM component laminin as a substrate, a specific CAM receptor called integrin on the growing processes will recognize this permissive macromolecule (Tucker, Rahimtula, and Mearow, 2006). After the protein binds to the integrins, the signaling mechanism of laminin-mediated neurite growth is then carried out by the protein kinase C (PKC) system. Inhibition of PKC results in decrease of neurogenesis and neurite regeneration (Freire et al., 2002; Luckenbill-Edds, 1997). As a result, neurons respond to laminin accordingly by attaching to the substratum and extending growth cones (Clark and Connolly, 1993; Dertinger et al., 2002; Hammarback et al., 1985).

In this study, we used a patterned substrate technique to observe the influence of laminin on the activity of chicken (*Gallus gallus*) sympathetic neurite growth by using laminin-free and dried laminin subregions as control settings and fresh laminin subregion as experimental setting (Letourneau, 1975; Luckenbill-Edds, 1997). The denaturation of dried laminin prohibits it from binding to the integrin on the cell surface and promoting neuritic outgrowth. On the other hand, with functional laminin protein, neurite extensions should be stimulated and maintained in vitro. In this study, I hypothesized that neurons would have more neurite growth on fresh laminin subregion than those on the laminin-free and dried laminin subregions. The cells would be observed by using a phase microscopy and the data would be collected by taking single still images periodically.

Materials and Methods

Dissection and poly-lysine treatment

Chicken (*G. gallus*) DRG and sympathetic chain were obtained by dissecting the 10-day chicken embryo followed by the procedures of dissection part 1 and 2 in Morris (2013). The cells were treated with trypsin and suspension in order to dissociate into single cells. Detailed steps were found in Dissociation of ganglia part 1 (Morris, 2013). Two coverslips were cleaned for laminin treatment (Morris, 2013). Both coverslip were coated with poly-lysine by following the coverslip treatment part 1 and 2 in Morris (2013).

Laminin Treatment and plating cell culture

Sterile pasteur pipette was prepared for laminin treatment (Morris, 2013). A laminin drop was dripped in the

middle of the two coverslips. The laminin drop in one coverslip was dried up before the next step. For the other coverslip, the laminin drop was kept wet and continued to the next step immediately. The coverslip with dried laminin served as the control group and the coverslip with fresh laminin served as the experimental group. The two laminin-treated coverslips were placed into growth medium by following the procedure of coverslip treatment 4 in Morris (2013). The cell suspension was plated onto the coverslip and the cultures were put into the incubator at 37 Celsius degrees for one week in order to allow the neurons to grow (Morris, 2013).

Measurement and data analysis

The two cell cultures were made into chip chambers and observed by using a phase microscopy (Nikon Eclipse E200 microscope) under 40X magnification followed by the procedure from Morris (2013). Single still images of every neuron were taken by the same microscopy with SONY Digital Interface DFX-X700 camera and software BTV version 6.0b1 on a Macintosh Desktop. Two trials of observation and measurement were conducted over the next two weeks. There were three subareas of the coverslips: laminin-free, dried laminin, and fresh laminin. The number of neurons in each subarea was recorded. And the number of neurons that had any unambiguous neurite growth, which means any process that is at least half of the cell body's diameter with parallel extension sides that is 1/3 wide of the cell body, was also noted. The percentage of neurons with neurite growth in each subarea was then calculated for further comparison.

Result

The focus of this experiment was the effect of laminin on neurite activity. Although neurons were allowed to grow in vitro for a week, not as many neurons as expected were found in the cultures. However, the amount of cells was still sufficient enough to conduct measurement and produce analyzed data for further interpretation.

The results indicate that comparing to the non-laminin region, there is an increased percentage of neurons with extensions on subarea of fresh laminin while only a slightly higher percentage on dried laminin subarea. The boundaries at the edge of the laminin-treatment areas on both coverslip could be observed under the microscope (Figure 1); therefore, the number of neurons in each subregion was easy to count.

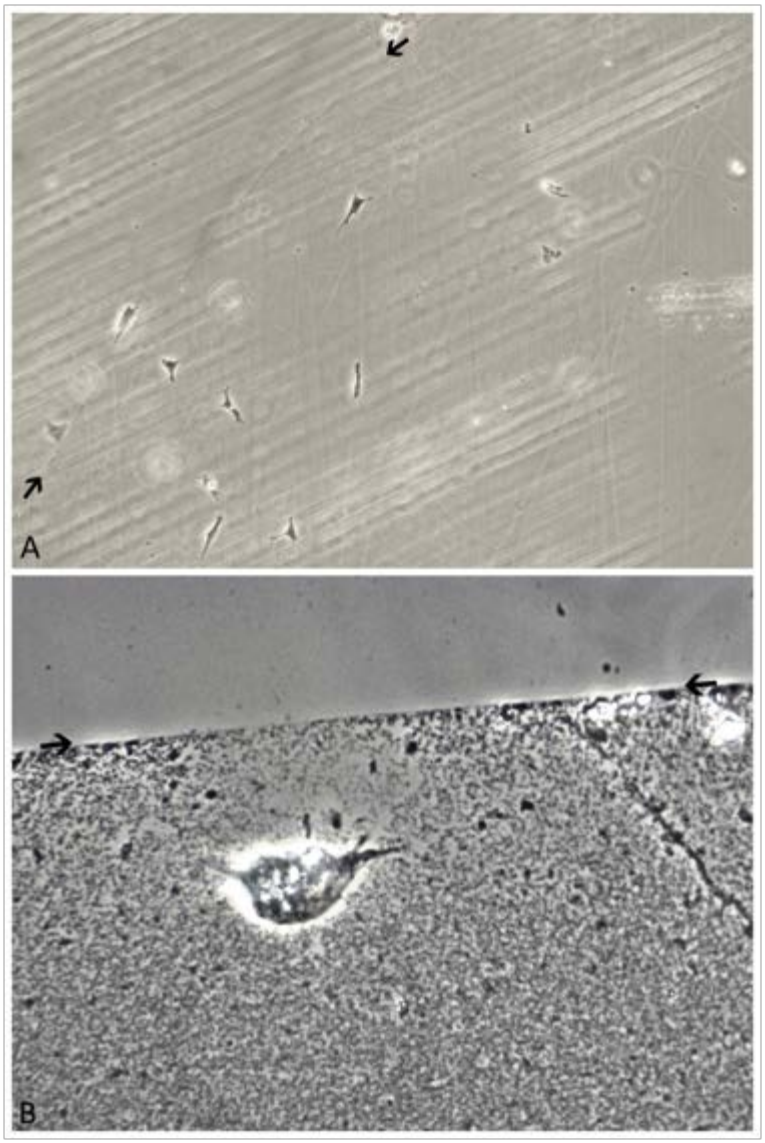

Figure 1: Neurons on laminin-free, dried laminin, and fresh laminin regions with borderlines indicated. (A) The two arrows indicate the borderline of no laminin treatment (left) and the dried laminin drop (right). (B) The borderline of laminin-free region (up) and fresh laminin region (down) is indicated by the arrows.

Neurons with extended cell shape but did not have clear processes were not considered as cells with neurite growth. Only neurons with unambiguous neurites that had length as half of the soma body and parallel sides of diameter that is 1/3 wide of the cell body were counted as cells with neurite growth (Figure 2).

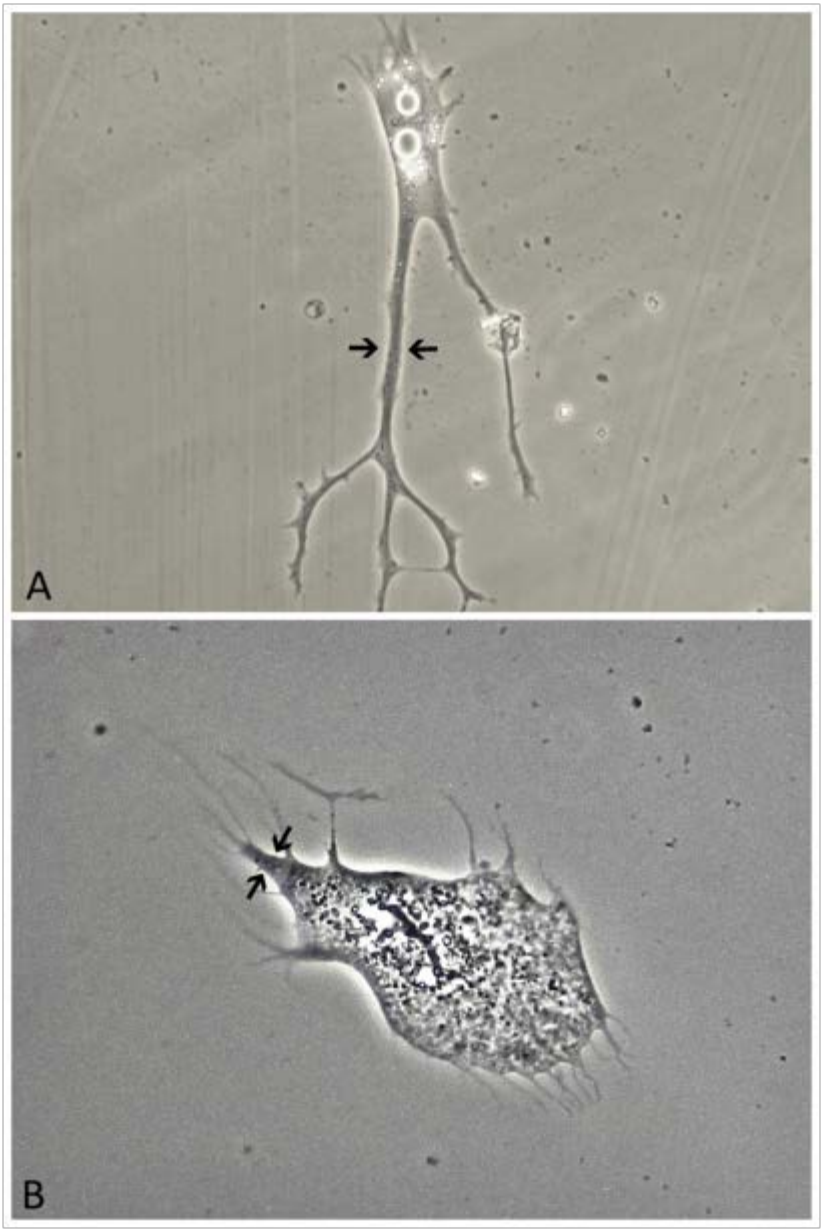

Figure 2: Neurons with unambiguous and ambiguous neurite outgrowth. (A) The neuron shows clear processes (pointed by arrows) that are at least half the length of the cell body and the sides of these extensions are parallel to each other. (B) Although the neuron demonstrates a potential axon development on the left side of the soma body (pointed by arrows), the shape of extension is ambiguous and it does not qualify for the description of a neurite in this study.

For the laminin-free subregion, there were only 30% of the cells that showed neurite growth. A 10% increase of neurons was observed in the dried laminin area. For fresh laminin region, the number of neurons with neurite growth was doubled (60%) than it was in the laminin-free area (Figure 3).

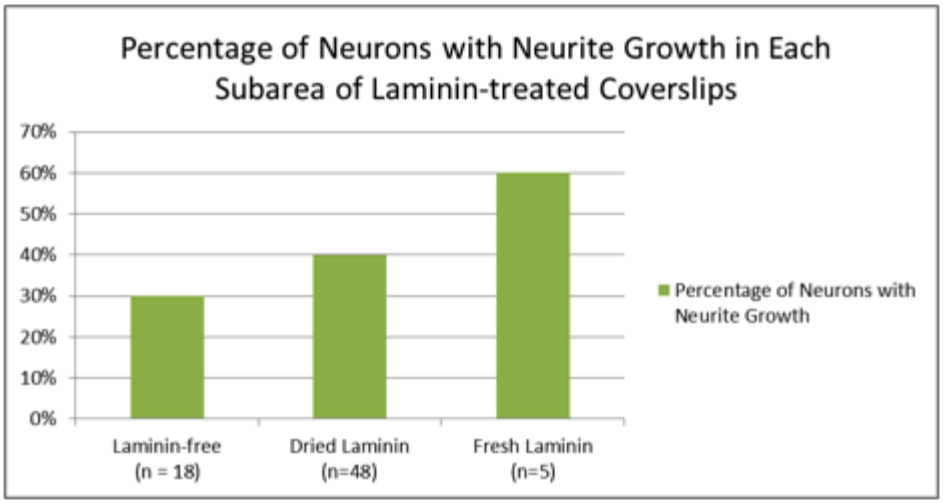

Figure 3: Percentage of neurons having process development in laminin-free, dried laminin, and fresh laminin subareas. The figure shows an increasing trend on percentage of neurons that develop processes from laminin-free region, dried laminin region, and to fresh laminin region.

Conclusion

In this experiment, the effect of laminin on neurons was studied. As a permissive substrate, laminin should aid the adhesiveness of neurite extension and allow processes to grow in vitro. Previous researchers have hypothesized that as a permissive ECM cue, laminin has an essential role on guiding nerve process development. By binding to the receptors on the growing tip of growth cones, laminin stimulates the neurites' pathfinding activities and help these extensions to attach on the substratum and grow in vitro (Bonnor and O'Connor, 2001; Luckenbill-Edds, 1997; Raper and Manson, 2010; Tessier-Lavigne and Goodman, 1996). The results of the study support my hypothesis, suggesting that the neurons in fresh laminin region would have more neurite growth than those in the control group (the laminin-free and dried laminin areas). If this pattern were consistently observed after many trials, then it will confirm that laminin serves as a strong promoter and guidance cue for neurons to perform neuritic extension in vitro.

Interestingly, the dried laminin also slightly promoted the outgrowth of nerve extensions. There is a 10% difference of neurons that develop neurites from laminin-free region to dried laminin region (Figure 3), suggesting that there was a potentially positive effect of dried laminin on neuron growth. According to Luckenbill-Edds (1997), laminin is a large, cross-shaped, multidomained protein composed by three polypeptide chains - an α chain, a β chain, and a γ chain. Two short arms are formed by the β and γ subunits while the long arm is consisted by the α subunit. Having a multidomained structure, specific domains and the polypeptide section within are associated with different cellular functions and behaviors (Luckenbill-Edds, 1997). In addition, to prototype laminin-1, there are many laminin isoforms that exist. These isoforms are all heterotrimers with different forms of α, β, and/or γ subunits. They have been modified to serve various functions and are found in different locations and species (Luckenbill-Edds, 1997). In this experiment, perhaps there is still part of the dried laminin that did not lose its conformation completely and could still bind to the receptors on the cell surface. In such a way, the denatured laminin could ultimately still promote neurite regrowth to a lesser degree than the non-denatured laminin.

The total number of neurons that grew on the coverslips was not as many as expected. Therefore, in order to refine this study for a more accurate outcome, we should increase the cell density that we plate out per coverslip. By doing so, we will observe a greater number of neurons, providing a larger data pool to analyze.

Previous research suggests that laminin shows extensive promotion on neuritogenesis in acidic conditions. According to Freire and Coelho-Sampaio (2000), acidification at a certain level does not fully denature laminin but induces a conformational change that maintains its tertiary structure and helps to accelerate its polymerization in vitro. Therefore, for a further understanding of the small positive effect on neurite growth by the dried laminin that may be denatured, neurons should be treated with laminin in various pH conditions in order to observe the effect of this neurite-promoting factor when its protein conformation is altered.

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