Promotion of neurite outgrowth on multi-molecular gradients by modulating downstream Rho pathways

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Introduction

In both the development and post injury environments in the central nervous system, an axon must accurately integrate multiple permissive and inhibitory guidance cues. Such cues have been incorporated into biomaterial platforms designed to model and study axon growth in these complex environments. Using photolithography and microfluidic techniques with poly(dimethyl siloxane) (PDMS), we can generate an *in vitro* microenvironment to present multiple cues and observe neurons' decision-making processes during neurite outgrowth. Our lab has characterized neuronal growth in response to gradients of multiple cues.

In this study, we aim to further elucidate the intracellular pathways by which growth cones integrate responses to both permissive and inhibitory cues presented by substrate bound molecular gradients, and to overcome the negative regions of the substrate by inhibiting downstream targets of signaling pathways. Gradients of the neurite outgrowth inhibitor chondroitin sulfate proteoglycan (CSPG) were formed in opposition to gradients of the growth-promoting extracellular matrix glycoprotein laminin-1 (LN). Neurons were treated with or without protein inhibitors to evaluate the role of candidate proteins in intracellular neurite guidance signaling mechanisms when presented with such gradients.

Methods

Immobilized gradients were generated by flowing protein solutions through a gradient mixer for physical adsorption of proteins on the substrate, as described by Dertinger et al[1]. The gradient mixer was made by transferring a template onto a silicon wafer using standard photolithography techniques. This was then used as a mold to make 3mm thick PDMS substrates. PDMS was adhered to glass after plasma oxidation of both surfaces.

Substrates were coated with poly-L-lysine (pLL) for 2 hours to allow subsequent protein adsorption. Gradients of the inhibitory proteoglycan CSPG (10μg/mL) were generated against gradients of the permissive glycoprotein LN (50μg/mL). Protein solutions were pumped through the channels at 0.2μL/min overnight, flushed with PBS, and the substrates were separated and stored in PBS until plating.

Dorsal root ganglia (DRG) were harvested from neonatal rats (P0-P2), trypisinized, dissociated, seeded at 25,000 cells/mL and incubated at 37°C for 24 hours. Pharmacological inhibitors to Rho Kinase (Y27632, 10μM) and Protein Kinase C (Go6976, 100nM) were added to culture media 1 hour after plating.

Substrates were immunostained sequentially with appropriate antibodies (CS56 and anti-LN) to visualize the two protein gradients, to ensure the preservation of the immobilized gradients on the substrate after treatment with pharmacological inhibitors. Samples were immunostained with a primary antibody, rinsed, and incubated with an appropriate secondary antibody. For controls, samples were processed without incubation with primary antibody. Density calibrations were performed using OpenLab image analysis software to quantify the amount of relative adsorbed protein on a normalized scale from 0-100.

For quantification of cell response, samples were immunostained using primary antibody RT97 directed against neurofilament with the appropriate secondary antibody. Phase contrast and fluorescent micrographs were captured with a Hamamatsu Orca ER camera and analyzed with MetaMorph software.

Cell response to the gradients was evaluated by quantifying adhesion along the width of the channel, neurite length, and neurite orientation. Treated samples were compared to control samples of neurons grown on identical gradient substrates without pharmacological treatment and neurons grown on uniformly coated LN and CSPG substrates.

Results and Discussion

Substrate-bound linear gradients of LN and CSPG were generated on channels of 250μm width and confirmed by immunohistochemistry and intensity measurements. DRG neurons exhibited good viability and healthy morphology on gradient substrates.

Neurons were treated with inhibitors to proteins known to be involved in the CSPG integration pathway. Without inhibitor treatment, cells showed a preference for higher LN concentrations and avoided higher CSPG concentrations in both adhesion and neurite outgrowth. Treatment with either inhibitor attenuated these effects—neurons showed less preference over either end of the gradient with regard to adherence and neurite length and orientation.

These studies begin to determine the mechanisms by which growth cones integrate guidance cues in the context of inhibitory and permissive gradients. In the future, additional inhibitors will be used to further evaluate these intracellular pathways. These results represent an important step towards establishing a biomaterial platform for investigating the multiple cues that are present in the microenvironment of developing neurons.

Acknowledgements

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References

1. Dertinger SK, et al. Gradients of substrate-bound laminin orient axonal specification of neurons. Proc Natl Acad Sci U S A 2002;99:12542.