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Co-immobilization of semaphorin3A and nerve growth factor to guide and pattern axons

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ABSTRACT

Immobilization of axon guidance cues offers a powerful tissue regenerative strategy to control the presentation and spatial location of these biomolecules. We use our previously developed immobilization strategy to specifically tether recombinant biotinylated nerve growth factor (bNGF) and biotinylated semaphorin3A (bSema3A) to chitosan films as an outgrowth and guidance platform. DRG neurite length and number for a range of single cues of immobilized bNGF or bSema3A were examined to determine a concentration response. Next single and dual cues of bNGF and bSema3A were immobilized and DRG guidance was assessed in response to a step concentration change from zero. Overall, immobilized groups caused axon extension, retraction and turning depending on the ratio of bNGF and bSema3A immobilized in the encountered region. This response indicated the exquisite sensitivity of DRG axons to both attractive and repulsive tethered cues. bSema3A concentrations of 0.10 and 0.49 ng/mm^2, when co-immobilized with bNGF (at 0.86 and 0.43 ng/mm^2 respectively), caused axons to turn away from the co-immobilized region. Immunocytochemical analysis showed that at these bSema3A concentrations, axons inside the co-immobilized region display microtubule degradation and breakdown of actin filaments. At the lowest bSema3A concentration (0.01 ng/mm^2) co-immobilized with a higher bNGF concentration (2.16 ng/mm^2), neurite lengths are shorter in the immobilized area, but bNGF dominates the guidance mechanism as neurites are directed toward the immobilized region. Future applications can pattern these cues in various geometries and gradients in order to better modulate axon guidance in terms of polarity, extension and branching.

Statement of significance

Nervous system formation and regeneration requires key molecules for guiding the growth cone and nervous system patterning. In vivo these molecules work in conjunction with one another to modulate axon guidance, and often they are tethered to limit spatial distribution.

The novelty of this research is that we provide a specific attachment method to immobilize an attractive signal, nerve growth factor, along with an inhibitory cue, semaphorin3A, to a substrate in order to analyze the interplay of these proteins on axon guidance responses. The scientific impact of this manuscript is that we show that dual-cued platforms are necessary in order to finetune and tailor specific axon responses for varying neuronal regenerative purposes.

1. Introduction

Axon pathfinding is a vital neuronal process for proper formation and patterning of the nervous system as well as innervation and recovery after nervous system injury. Thus, anatomical development and regeneration is heavily dependent on localized expression of guidance molecules in order to facilitate axon guidance mechanisms. The growth cone, the leading edge of the axon, will detect permissive and inhibitory signals from soluble and substrate-bound molecules in the extracellular environment and subsequently direct the axon along its intended path. Once bound to growth cone surface receptors, guidance signals are then transduced by cytoplasmic signaling pathways that promote cytoskeletal rearrangement and directed motility and growth [1]. These biomolecules, present in the surrounding microenvironment, often work in concert with each other to achieve directional efficiency of neuronal guidance to the appropriate targets (for reviews see [2,3]).

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Two families of neuronal proteins especially proficient in chemotaxis and haptotaxis include the semaphorins and the neurotrophins. Semaphorins are considered mainly a repulsive cue since, once bound with a semaphorin receptor neuropilin, they can induce a collapse in the growth cone and thereby effectively prevent any opportunity for further neurite growth [4]. In contrast, the neurotrophin nerve growth factor (NGF) is considered a chemoattractant because of its ability to initiate neurites while enhancing extension after protein–cell receptor interaction. The cell receptors that activate this process are p75 neurotrophin receptor (p75NTR) and tropomyosin receptor kinase A (TrkA) receptor [5,6]. A suggested synergism between attractive and inhibitory cytokines indicates the possibility to exhibit greater control on axon guidance and proper patterning [7], which is vital to tissue repair after nervous system injury. This is evident with netrins (permissive) and slits (inhibitory) that are responsible for the patterning and development of spinal cord tracts [8,9]. Many other guidance proteins (such as chondroitin sulfate proteoglycans (CSPGs), ephrins, netrin-1, semaphorin3D) direct retinal ganglion axons from the eye to the brain (for review see [10]). Additionally, NGF and semaphorin3A (Sema3A) play a major role in regulating sensory neuron extension and pathfinding [11–13]. Therefore, this endogenous distribution of permissive and inhibitory guidance factors, specifically NGF and Sema3A, motivated us to examine the dual effects these biomolecules implement on sensory neuron guidance.

In the living environment haptotaxis plays a significant role in many cellular functions, ranging from morphogenesis and embryonic development to regenerative processes [14]. Immobilization of biomolecules has been used as a strategy to decrease dosing requirements for tissue-regeneration by increasing sustainability of the molecule as well as better modulation of orientation and spatial location of these factors (for review see [15]). Additionally, immobilization of factors is found endogenously largely through protein sequestration by extracellular matrix molecules, which serves to enhance biomolecular responses as well as cell recruitment to areas of trauma [16,17]. The strong interactions associated with immobilization of growth factors and proteins have elicited significantly better and appropriate cellular responses compared to adsorbing the protein to a biomaterial substrate [18,19]. Based on this evidence, immobilizing guidance molecules is our choice strategy in modulating axon outgrowth and guidance.

For this study, we use a previously reported immobilization method using a heterobifunctional crosslinker, a maleimide–streptavidin molecule and recombinant biotinylated proteins, NGF and Sema3A [19]. Building upon these findings, the focus and novelty of this study is to examine axon guidance responses to co-immobilized uniform concentrations of NGF and Sema3A. This is accomplished by (1) determining concentration effects within the region to either immobilized NGF or Sema3A through neurite outgrowth and number assessment and (2) analyzing axon guidance (neurite outgrowth/inhibition and turning) when DRGs are cultured outside of and near regions containing both immobilized NGF and Sema3A. From this study, we discover how DRGs respond to ever-present and encountered co-immobilized cues of NGF and Sema3A. We compare these findings to previous experiments utilizing both soluble and immobilized NGF and Sema3A as outgrowth and guidance methods. Additionally, these findings expand our immobilization strategies to modify and propel our guidance model into future regenerative applications.

2. Materials and methods

2.1. Synthesis of heterobifunctional crosslinker and guidance proteins

N-(2-mercaptoethyl)-3-(3-methyl-3H-diazirine-3-yl) propanamide (N-MCEP-diazirine) was synthesized in the dark as we have reported previously [19]. Briefly, 4-oxopentanoic acid (Sigma, St. Louis, MO, USA) and cysteamine (Sigma) were refluxed overnight at 120 °C. The product was purified by flash chromatography and freeze-dried. For the addition of the diazirine component, the procedure was completed in the dark because low wavelengths can degrade intermediates and the final product. First, the freeze-dried product was refluxed with 3% ammonia solution (J.T. Baker, Phillipsburg, NJ, USA) at 4 °C for 5 h. The reaction was reduced to −78 °C and hydroxylamine-O-sulfonic acid solution (Sigma) was gradually added and stirred for an additional 5 h. The reaction continued overnight to allow excess ammonia to evaporate. This solution was then washed with CH_3OH (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA) and triethylamine (Sigma) was added. The mixture was cooled to 0 °C, titrated with I_2 solution (Sigma) and purified again by flash chromatography.

2.2. Immobilization of bNGF and bSema3A

bNGF (29 kDa) and bSema3A (91 kDa) were synthesized as we have reported previously [19,20]. To immobilize these proteins, chitosan films were prepared [19]. Briefly, chitosan (Novamatrix, Sandvika, Norway) was dissolved at 2 wt% in acetic acid and 3 mL/well was pipetted into a 6 well-plate, for co-immobilization guidance studies, or 0.5 mL/well in a 48-well plate for protein concentration analysis. The plates were dried in a fume hood for 24 h, neutralized with an ammonium hydroxide solution (NH_4OH: ddH_2O:CH_3OH at 3:7:90) and washed in phosphate buffered saline (PBS, pH = 7.4). First, the diazirine ring on the N-MCEP-diazirine was attached to the primary amines of the chitosan film at 25 mM when exposed to UV light (365 nm, 8 W, 2.7 mW/cm^2) for 10 min. Films were then washed in PBS (pH = 7.4) with 50 mM dithiothreitol (DTT, Chem-Impex International) and rinsed with PBS (pH = 7.4). Next, maleimide–streptavidin (mal-strep, 0.2 μM, Sigma) was attached to the crosslinker for at least 2 h at room temperature in PBS containing 20 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP, pH = 7.4, Chem-Impex International). After washing films in PBS again, the biotinylated proteins were added at their respective concentrations overnight at 4 °C for each experiment described below. Using a 16- or 18- gauge needle and syringe, films were extensively washed in 20 mM Tris–hydrochloride + 150 mM NaCl + 0.1% Tween 20 (TBST, pH = 7.5), the plates were rocked for 1 h, and then the process was repeated two more times. Films were finally rinsed in PBS (pH = 7.4).

2.3. Concentration response of bNGF and bSema3A on DRG outgrowth

For cell studies, films were sterilized in 70% ethanol for 30 min. Laminin (Life Technologies, Grand Island, NY, USA) was adsorbed to the films for at least 3 h at room temperature at a final concentration of 5 μg/mL before the proteins were immobilized. The same protein immobilization procedures were performed under aseptic conditions for DRG outgrowth studies.

DRG extracts were obtained from 9 d chick embryos, cleaned of any debris, and set into wells containing serum free medium (13.4 g/L DMEM (Dulbecco's Modified Eagles Medium), 1.176 g/L sodium bicarbonate, 1% L-glutamine (Life Technologies), 1% streptomycin–penicillin (Life Technologies), 5 μg/mL human insulin, 5 μg/mL transferrin, 3×10^{-8} M sodium selenite, 100 μM putrescine, 2×10^{-8} M progesterone, all Sigma) [19]. These culture parameters containing non-serum medium has shown to reduce non-neuronal populations after DRG isolation [21]. The addition of NGF enhances DRG survival compared to the medium alone and has been used as a standard for outgrowth and guidance studies, with 10–20 ng/mL being the optimal concentration range for embryonic day 15 DRG neurons [22]. Therefore, soluble NGF will be added as a positive control parameter for these studies. DRG