High Throughput Screening To Identify Novel Molecular Pathways Involved In Neurite Outgrowth

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Abstract

An abundance of data has been generated with the completion of the genome project which provides a comprehensive view of kinases and the molecular regulators of the cell. However, very little is known about the mechanism of these regulators and their relationship to molecular pathways responsible for the development of complex three-dimensional tissues. To acquire a better understanding, some labs have already employed a combination of functional genomics (loss of function) screen with morphological analysis in explant cultures. Here, to discover novel kinase regulated molecular pathways in neurite outgrowth we have used Neurite Outgrowth Assay for High Content Screening to screen for gain or loss of neuritis in neuronal cells exposed to siRNA kinome. Furthermore, neurite outgrowth was quantified using Neurite Outgrowth Quantification Kit. To that end, we have shown that neurite outgrowth in N1E-115 neuroblastoma cells was significantly stimulated in Millicell® inserts coated with the ECM protein laminin compared to BSA controls (*p<0.05; n=5). Furthermore, application of 75μM nocodazole, a microtubule-destabilizing drug, reduced the formation of neurites. We then quantitated the effect of knocking down 18 different kinases on neurite outgrowth. The characterization of neurite formation, maturation and collapse/resorption is an area of intense interest, since these cellular processes are essential for interconnection of neuronal cell bodies. Therefore, major efforts in central nervous system drug discovery research are focused on the identification of compounds that affect neurite outgrowth. In summary, our results demonstrate an unbiased approach in understanding the relationship of molecular pathways and their impact on morphological parameters in cellular systems.

Introduction

Proper neuronal migration and establishment of circuitry are key processes for nervous system functioning. During development, neurons extend numerous processes that differentiate into dendrites and axons. These processes, also termed neurites, are critical for communication between neurons.

Neurites are particularly interesting in relation to neuropathological disorders, neuronal injury & regeneration, and neuropharmacologic research and screening. Nerve transection in the mammalian central nervous system (CNS) was once believed to be irreversible, however, it has recently become apparent that the inability of damaged nerve fibers to regenerate is an active process under the control of molecules able to inhibit and repulse growing neurites.

Many of these key molecular regulators are kinases. However, very little is known about the mechanism of these regulators and their relationship to molecular pathways responsible for appropriate differentiation and survival of neurons. It is becoming apparent that a set family of kinases and their downstream targets have a major function in regulating CNS development, such as the Rho family of small GTPases and Pak family of kinases. Furthermore, kinases regulate neuronal polarity, morphology, migration and synaptic function. Accordingly, there are also strong evidence that certain kinases are required for normal cognitive function. Case in point, the progression of Alzheimer's disease has been associated with the loss of Pak1. Together, these new and motivating findings promote the examination of the function of other kinases in the nervous system, thus, paving the way for novel strategies towards better understanding, improved diagnosis and therapeutic treatment of diseases that affect the CNS.

Methods

Neurite Outgrowth Assay Protocol using N1E-115 cells

- Culture cells in maintenance media until ~60-70% confluent.
- Replace maintenance media with Millipore differentiation media (NS002) for 24 hours.
- Coat underside of Millicell® 12 well inserts with 400 μL of 10 μg/mL Laminin (Millipore, AG56P) or 10 μg/mL BSA (Millipore, 20-191) (negative control) for 2 hours at 37˚ C.
- Use enzyme free dissociation buffer to remove cells from flask. Centrifuge and count cells. Resuspend cells in differentiation media at a concentration of 100,000 cells/100 μL (1 x 10^6 cells/mL).
- Following coating, remove each insert from its coating solution (rinsing not required) and place into a new well of a 12 well plate containing 1200 μL of differentiation media.
- Add 300 μL of cell suspension to the top of the insert.
- Add 75μM nocodazole or 25μL of adenovirus containing siRNA kinome.
- Incubate plate at 37˚ C for 48 hours.
- Following the neurite extension period, remove each insert from the culture well, gently pipette off the liquid from the top of the membrane, and place into 1200 μL of PBS in a new 12 well plate.
- Transfer insert to a new well containing 400 μL of -20˚ C methanol.
- Fix for 20 minutes at room temperature.
- Rinse briefly in 1200 μL of PBS.
- Place insert into 400 μL of Neurite Stain Solution (90242).
- Stain for 15-30 minutes.
- Rinse briefly in 1200 μL of PBS.
- Swab cell bodies from top of insert and rinse briefly in 1200 μL of PBS. Continue rinsing as needed.
- Place swabbed inserts into 15 mL BDFalcon™ (round-bottom) tubes.
- Add 100 μL of Neurite Stain Extraction Buffer (90243) to the top of each insert.
- Incubate for 5 minutes at room temperature.
- Spin inserts at 1200 RPM for 1 minute.
- Read ~75 μL of each sample at an absorbance of 590 nm.

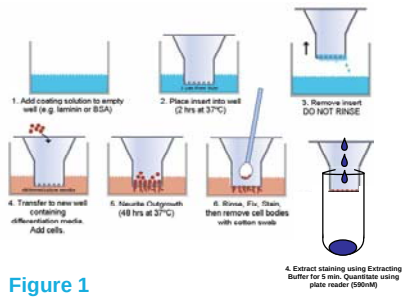

Figure 1

Overview of Neurite Outgrowth Assay Protocol

Results

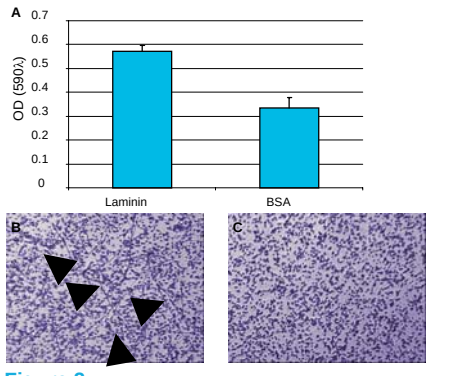

Figure 2

Stimulation of neurite outgrowth from N1E-115 cells in response to a range of membrane coating substrates. A) Neurite outgrowth was significantly stimulated in Millicell® inserts coated with the ECM protein laminin (10 μg/mL) compared to BSA controls (10μg/ml) (*p<0.05; n=5). B) Image of N1E-115 neurites in Millicell® Laminin-coated inserts (10 μg/mL). C) Image of negative control. BSA-coated inserts (10 μg/mL) did not induce neurite growth of differentiated N1E-115.

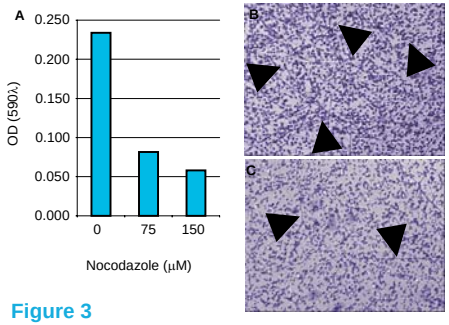

Figure 3

Inhibition of neurite outgrowth from N1E-115 cells in response to the potent microtubule depolymerizing agent nocodazole. A) Neurite outgrowth was inhibited in cells exposed to nocodazole compared to control. (OD reading normalized to BSA negative control). B) Image of N1E-115 neurites in Millicell® Laminin-coated inserts (10 μg/mL), C) Image of N1E-115 cells treated with 75μM nocodazole in Millicell® Laminin-coated inserts

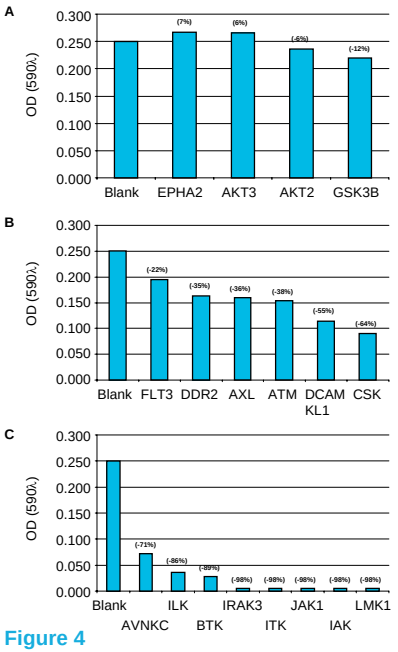

Figure 4

Neurite outgrowth from N1E-115 cells in response to different kinase siRNA adenovirus. Data represents kinase siRNA adenoviruses that A) did not change B) moderately and (C) drastically decreased neurite outgrowth. %Change in parenthesis (OD reading normalized to BSA negative control).

Summary

- Neurite outgrowth was significantly stimulated in Millicell® inserts coated with the ECM protein laminin (10 μg/mL) compared to BSA controls (10μg/ml)
- Neurite outgrowth was inhibited in cells exposed to nocodazole compared to control
- N1E-115 exposed to kinase siRNA adenoviruses were divided into 3 categories:
  - No change in neurite outgrowth
  - Moderately decreased neurite outgrowth
  - Drastically decreased neurite outgrowth
- Surprisingly, of the kinases that we tested none of them significantly increased neurite outgrowth