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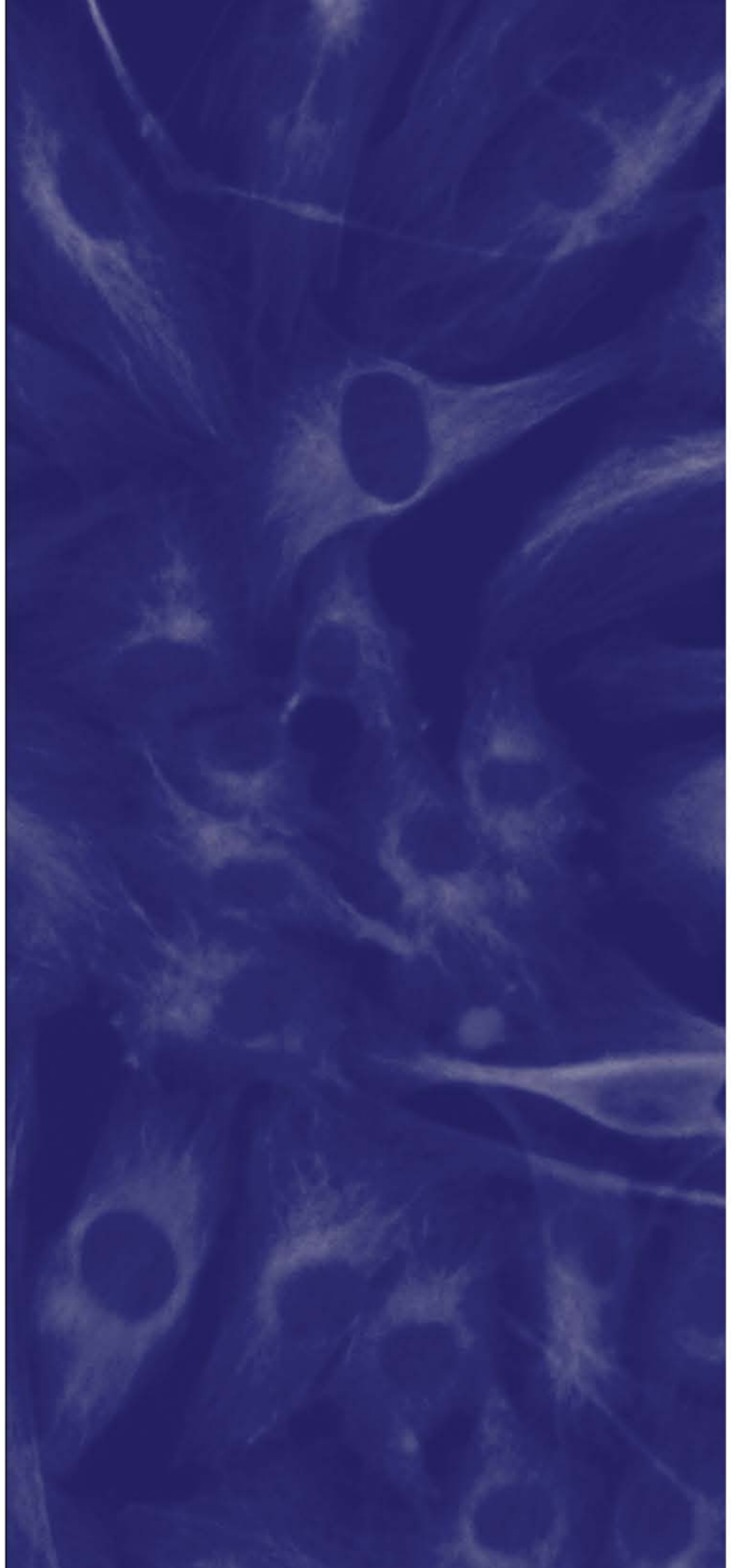

Neuronal Morphology and Neurite Outgrowth Characterization Using an Automated Image Analysis System

Amy M. Peters, Everett Ramer, Oleg Lapets,
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Abstract

In vitro neuronal assays offer ease of manipulation, scalability, and ability to understand compound effects at the cellular level. We have validated a neurite outgrowth assay using fluorescent reagents and image analysis systems that provide these benefits as well as the ability to automatically and objectively analyze the results for more robust assays.

NeuroScreen-1 cells were treated with nerve growth factor to determine functionality of neurite outgrowth. Neurite characteristics such as count, maximum length, and degree of branching, as well as changes in neuronal morphology were evident in the treated wells. Primary rat cortical neurons were tested with multiple compounds to determine what effects they had on various neuronal properties. SU6656 exhibited decreases in neuronal cell number and reduction of neurite length, while maintaining neuronal morphology (shape). Validation of primary cultures also ensured that non-neuronal cells did not interfere with the measurement, accurately reporting the percentage of neuronal- and non-neuronal cells in the cultures.

Introduction

Abnormalities of neuronal cells are implicated in a variety of pathophysiological conditions such as Alzheimer's and Parkinson's disease. These disorders affect changes in neuronal cell morphology and/or changes in neurotransmitter expression. Mounting evidence associates neuronal cell death after stroke and in conditions following hypoxia, ischemia, blood trauma injury, etc. Some disease conditions affect outgrowth and elongation of neurites, branching of the neurites, changes in neuronal cell survival, cell body area, and expression of certain genes. Axonal outgrowth and functional recovery after nerve injury and the altering of various signaling cascades (including Raf/ MEK/MAP kinase pathways) may change the amount of proliferation and neurite growth within a cell (Das et al., 2004). Being able to multiplex these and other changes can help understand the pathways leading to abnormalities and how to treat them.

Neuronal cell morphology, including neurite outgrowth, total neurite count, cell body size, and growth cone behavior, is modulated by a variety of conditions such as trophic factors, electrical activity, synaptogenesis, and functional maturation and differentiation of neurons (Thoenen, 1991; Ooyen et al., 1995; Files and Nelson, 1992). It is these morphological changes than can help detect neurotoxins. Neurogenic regions in the adult central nervous system, such as the spinal cord, contain neural stem cells. Neural stem cells from these regions proliferate in response to factors, such as neurotrophins, epidermal growth factor, or basic fibroblast growth factor, and differentiate into neurons and glial cells *in vitro* (Gritti et al., 1996).

In order to evaluate changes in morphology, the Neuronal Profiling (NP) BioApplication, along with the Neurite Outgrowth HitKit® HCS Reagent Kit, were verified using Neuroscreen™-1 (NS-1) cells, a sub-clonal cell line of PC-12 cells (Cellomics, Pittsburgh PA). PC-12 cells are established as a standard model system for the study of neuronal cells (Greene et al., 1998; Tsuji et al., 2001; Wu and Bradshaw 1996) and acquire a number of properties characteristic of sympathetic neurons (Das et al., 2004). Neuroscreen-1 cells display several significant advantages over the parental PC-12 cell line, including a much shorter doubling time, easier growth and culture, a lower tendency to aggregate, and visible neurite outgrowth in 48 hours, compared to 6-7 days in PC-12 cells. Neurotrophic factors such as nerve growth factor cause the NS-1 cells to differentiate into neuronal-like cells with neurites within 3-4 days.

Primary neuronal cultures were used in this study to validate the assay and compare compound results obtained with cultured cells. Primary cultures will grow neurites within 5 - 7 days (without NGF treatment). Experimentation compared several morphological features of untreated wells to see if changes occurred when treated with possible inhibitors (SU6656, Bisindolymide-I and U0126) and agonist NGF. All testing was completed with the Cellomics ArrayScan® HCS Reader.

Materials and Methods

Materials

Cells and Plates:

- NS-1 cells were passaged at 70% confluency. Cells were grown in collagen-I flasks in RPMI-1640 media with 10% HS, 5% FBS, 1% P/S, and 1% L-Glutamine (Hyclone, Logan UT). Cells were then plated onto Collagen-I coated 96-well microplates (Becton Dickinson, Franklin Lakes NJ) to ensure attachment.
- Primary striatal and cortical neurons (Cambrex, Walkersville MD) were cultured in Primary Basal Neurite Media (PBNM) containing Single Quots (Cambrex) and immediately plated onto Poly-L lysine plates (Becton Dickinson).

Compounds and Stains:

- Neurite Outgrowth HitKit HCS Reagent Kit (Cellomics) was used for initial analysis. An additional TRITC secondary (DyLite 549 GAM, Pierce, Rockford, IL) was also used.
- Compounds (EMD BioSciences, San Diego, CA) selected were the following:
  - SU6656 - inhibitor of the Src-Family of Kinases; can inhibit axon outgrowth once induced by Netrin-1 and GDNF
  - U0126 – inhibitor of MEK1 and MEK2
  - Bisindolamide-I (Bis-I) – cell permeable inhibitor of PKC
  - 7S-NGF (NGF) – promotes neuron survival and neurite outgrowth
- Fixation was by using Formaldehyde (Fisher Scientific, Fair Lawn NJ) diluted to 3.7%

Methods

NS-1 testing of agonist vs antagonist

NS-1 cells were plated at 3000/well onto collagen I-coated plates and incubated overnight. Media was removed and cells were treated with combinations of NGF (final concentrations 0 - 800 ng/mL) and potential inhibitors SU6656 (0 - 20 µM), BisI (0-8 µM), and U0126 (0 – 50 µM). Plates were incubated for 72hr. After incubation, solution was removed and cells were fixed with 3.7% formaldehyde for 20 min. Plates were washed twice with Neurite Outgrowth Buffer (NOGB) and incubated at RT for 1 hr with kit primary. After washing plates twice with NOGB, cells were incubated at RT for 1 hr with Dyomics secondary Ab (549). Plates were again washed twice with NOGB, once with Wash Buffer, and sealed in Wash Buffer. Plates were run with the NP BioApplication at 10x, taking a total number of 300 cells per well.

Primary neuronal cultures – antagonistic studies

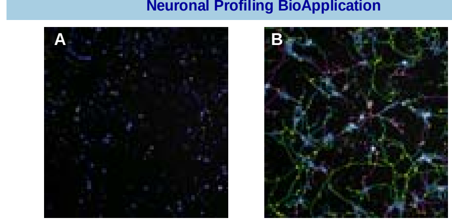
Primary striatal and cortical cells were purchased from Cambrex and plated following their protocol except for total volume per well (120 µl of cell media per well vs. 200 µl) decreasing the total cell number to 48,000/well in order to have more wells to evaluate. After incubation for 4 hr, media was removed and replaced with 1:2 dilutions of BisI (0 - 8 µM), SU6656 (0 – 20 µM), U0126 (0 – 50 µM), or NGF (0 - 400 ng/mL). Plates were incubated for 7 days, with a media change (same compounds and concentrations) on day 4. Solution was then removed and plates were stained using the kit protocol. Plates were run with the NP BioApplication at 5x, taking 9 fields per well.

Cellomics Neuronal Profiling BioApplication

The NP BioApplication quantifies morphological changes in neurons. It allows the user more control over selecting neurites based on differences in morphology and intensity, as well as selecting neurons based on parameters in the nuclear and neuronal channels. One can also identify subpopulations of cells, due to biology, by combining their features into events.

All nuclei (neuronal and non-neuronal) are identified in Channel 1 where nuclear features (size, shape, intensity, etc.) are measured. Neurons and neuronal-like cells are identified in Channel 2 with a primary antibody against a protein specific to neurons present in both neuronal cell bodies and neurites. Cell body (size, shape, and intensity) and neurite features (count, size, intensity, branch and cross point counts, etc.) are measured in Channel 2. Figure 1 is an example of overlays found in Channel 1 and 2, using primary cortical neurons stained with the Neurite Outgrowth Reagent Kit.

Figure 1. Identification of Channels and Overlays using the Cellomics Neuronal Profiling BioApplication



Images representing channels and overlays with the Neuronal Profiling BioApplication.
(A) Channel 1: **ValidNuclei** (blue) and **RejectedNuclei** (orange)
(B) Channel 2: **ValidCellBody** (blue), **RejectedCellBody** (red), **NeuritePoint** (yellow), and **Neurites** (green, teal, and pink). Differences in neurite colors is to help distinguish possession of the neurites to specific cell bodies.

The NP BioApplication allows the user to define and screen for occurrence of specific Events. The event feature used in these experiments was:

Event = [(Neurite Count) > threshold] OR [(Neurite Total Length) > threshold]

Results

NS-1 testing of agonist vs antagonist

Various morphological features were observed to see if Bis-I, SU6656, and/or U0126 inhibited the effects of NGF on NS-1 cells. Comparison of no NGF to any concentration of NGF only showed a mean increase in neurite count, length, area, and branch point; however, there was not a true dose response when looking at the different concentrations. Table1 gives an example of differences found between untreated and 200 ng/mL NGF.

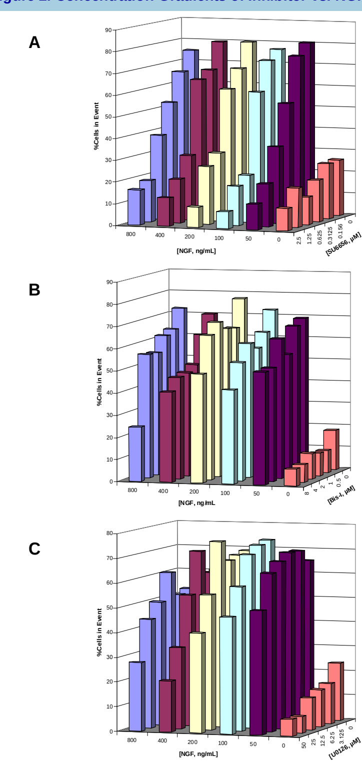
Table 1. Mean Feature Fold Differences of NGF

Data from triplicate wells of untreated vs 200 ng/mL NGF. Features represent mean values within the well (Mean) or the percentage of cells above a set threshold (%High).

Output Feature	NGF concentration (ng/mL)		Fold difference
	0	200	
Mean Neurite Total Length	26.51	163.50	6.17
Mean Branch Point Total Count	0.27	3.36	12.44
Mean Branch Point Distance From Cell Body	1.70	23.29	13.70
%High Cell Body Area	12.74	41.09	3.22

An event was set in the BioApplication to look at the percent of cells that had neurite count or neurite total length above basal levels (no NGF, no inhibitor). Decreases in this event was evident upon increasing doses of SU6656, Bis-I, and U0126 (Figure 2A, 2B, 2C).

Figure 2. Concentration Gradients of Inhibitor vs. NGF in NS-1 cells

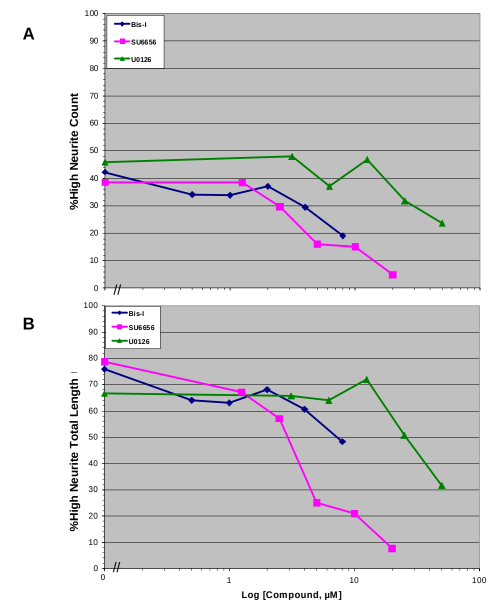


Bar graphs representing dose responses of NGF plotted against dose responses of (A) SU6656, (B) Bis-I, and (C) U0126 with respect to the selected Event, defined in the Methods section as the percent of cells expressing the value for Neurite Count OR Neurite Total Length above the reference well-determined levels.

Reference wells (without inhibitor) were analyzed to obtain threshold values for specific output features. The percent of cells that had values above this threshold, as well as the mean (average) value of all cells within a well were evaluated. Figure 3 demonstrates the breakdown of this Event into separate %High Neurite Count and %High Neurite Total Length of inhibitors against 200 ng/mL NGF.

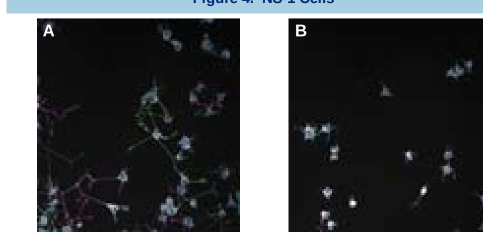
In addition, as the concentration of inhibitors increased, a decrease in neurite count, length, area and branch points were evident. At the highest dose of inhibitor, SU6656 had the largest effect over control, followed by Bis-I, and then U0126. Figure 4 represents images (with overlays) of NS-1 cells treated with NGF and NGF + inhibitor.

Figure 3. Neurite OutGrowth inhibition in NS-1 Cells



Graph representing %High Neurite Count and %High Neurite Total Length for varying doses of SU6656, Bis-1, and U0126 on 200 ng/mL NGF. In all three inhibitors, increasing doses caused decreases in the percentages of cells exhibiting each feature.

Figure 4. NS-1 Cells

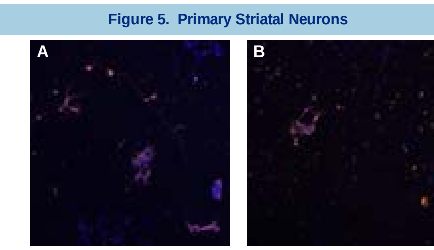


Images (10x) exhibiting overlays of NS-1 cells treated with (A) 800 ng/mL NGF and (B) 800 ng/mL NGF + 8 µM Bis-I after running on the ArrayScan with the NP BioApplication. Note the difference in neurite length, number, and branches in cells treated with inhibitor.

Inhibition of Primary Striatal Neurons

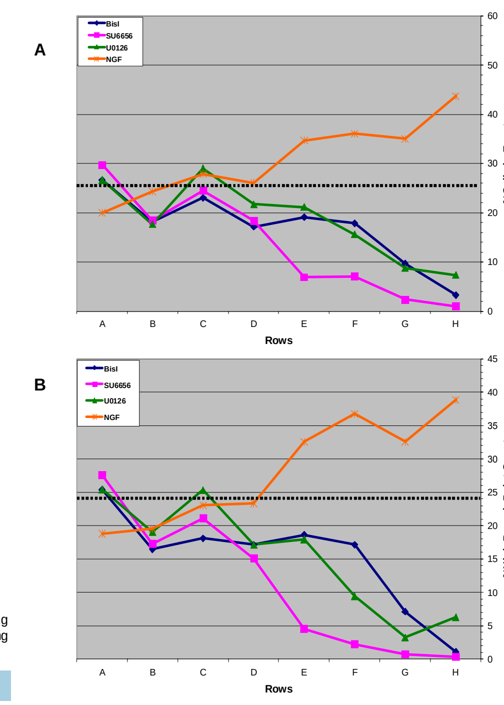
Primary striatal neurons were plated and incubated over 7 days to evaluate possible changes in neurite and morphological characteristics when treated with the above inhibitors and NGF. Figure 5A is a composite image of neurites that were treated with NGF; 5B is after treatment with Bis-I. Notice that the overall neurite length has decreased, as well as the number of neuronal cells. Figures 6A and 6B are a set of graphs representing % Event Cells (as mentioned in Figure 2) and % High Branch Point Count (% of cells with value above a set threshold), with increasing concentration of inhibitors decreasing most feature values and increasing NGF concentrations increasing these values. The average Cell Body area and average intensity of neurites did not change between inhibitors or NGF over untreated values (data not shown).

Figure 5. Primary Striatal Neurons



Images (5x) representing primary striatal neurons treated with (A) 400 ng/mL NGF and (B) 8 µM Bis-I. It is easy to see a larger number of neurons as well as more neurites in the cells treated with agonist vs. inhibitor.

Figure 6. Inhibitory Effects in Primary Striatal Neurons

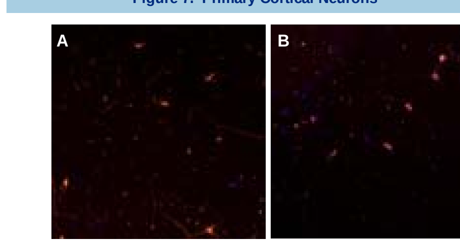


Graphs representing (A) the percent of cells exhibiting Event 1 and (B) percent of cells with values above a set threshold for total number of branch points. Thresholds were based off of reference wells consisting of untreated cells. Dashed line represents average value of untreated wells. In both instances, increasing concentrations of NGF caused an increase over basal levels (represented by a dashed line) for both % branch point and event. Increasing concentrations of inhibitor caused a decrease for both in a dose-dependent manner. Data represents duplicate wells per concentration, run on the ArrayScan with a 5x objective, 9 fields per well.

Inhibition of Primary Cortical Neurons

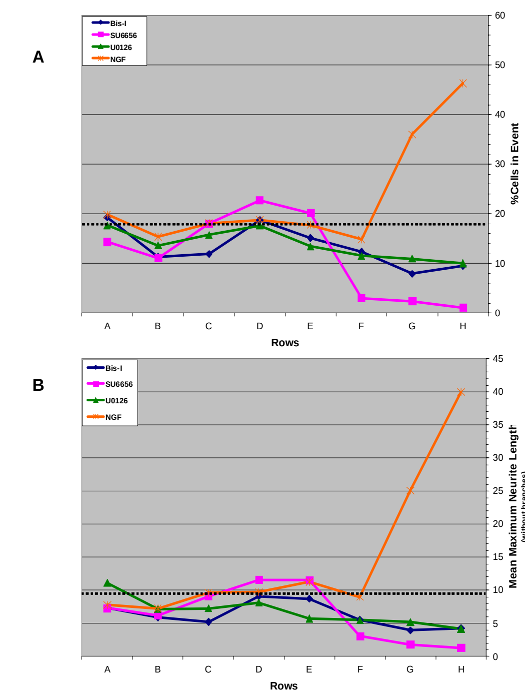
Primary cortical neurons were plated (similar fashion as primary striatal neurons) and incubated over 7 days to evaluate possible changes in neurite and morphological characteristics when treated with various doses of the above inhibitors and NGF. Figure 7A is a composite image of neurites that were treated with NGF; 7B is after treatment with SU6656. Note the change in number of neuronal cells as well as a decrease in neurite length. Figures 8A and 8B are graphs representing % Event Cells (as mentioned in Figure 2) and Mean Neurite Maximum Length (without counting branches), with the inhibitors decreasing these feature values as concentration increases, and NGF increasing these feature values as concentration increases. Changes in neurite characteristics were not as severe as in striatal neurons when comparing equal concentrations of inhibitor.

Figure 7. Primary Cortical Neurons



Images (5x) representing primary cortical neurons treated with (A) 400 ng/mL NGF and (B) 10 µM SU6656. Total number of neurons, as well as neurite count and length have decreased in wells treated with inhibitor.

Figure 8. inhibitory Effect in Primary Cortical Neurons



Graphs representing (A) percent of cells exhibiting Event 1 and (B) mean maximum neurite length (not including length of branches). Increasing concentrations of NGF caused an increase in % Event 1 and maximum length over basal levels, while increasing concentrations of SU6656, U0126 and Bis-I caused little change at early doses and decreases in higher doses. Dashed line represents average value of untreated wells. Data represents duplicate wells per concentration, run on the ArrayScan with a 5x objective, 9 fields per well.

Conclusions

NS-1 cells

- In NGF-only treated cells, increasing doses does not elicit a dose-responsive increase in neurite characteristics. Instead, an immediate increase occurs in the lowest concentration of NGF over untreated levels. Differences in neurite length, branch point count, and cell body area are evident between treated and untreated wells.
- Relative potency of compounds that inhibit neurite outgrowth of NGF are: SU6656 >> Bis-I >> U0126

Primary neuronal cultures

- Dose-dependent inhibition of neurite characteristics are similar as in NS-1 cells; however, some morphological differences (e.g., cell body area) are not different from basal levels (as was found in NS-1 cells).
- Relative potency of Bis-I on inhibiting neurite morphology in primary cortical neurons is not as effective at the tested concentrations as in primary striatal neurons.

The Neuronal Profiling BioApplication (in conjunction with the Cellomics HCS Readers and Neurite Outgrowth Reagent Kit) can easily detect and discriminate morphological changes in both primary neuronal cultures and cell lines.

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