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Evaluating neurite outgrowth and signal integration in response to NGF and GDNF in neuroblastoma cell lines

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Introduction

- Neuroblastoma is a cancerous tumor that develops from cells of the neural crest. We hypothesize that neural crest cells' failure to differentiate into nerve cells is an important step leading to neuroblastoma.
- Developing neurons rely on sustained signals from the local tissue to form and maintain innervation. This signal takes the form of neurotrophin ligands (like NGF and GDNF) which bind to receptors in the cell membrane.

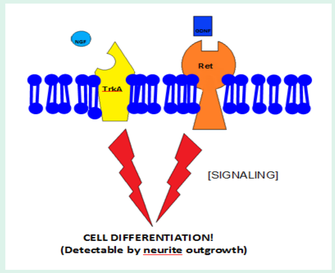
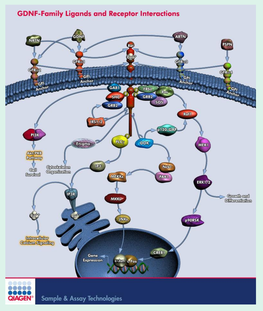


Figure 1. Simplified model of relevant cell signaling

- The roles of receptor tyrosine kinases (RTKs) in governing cellular processes like differentiation are extensive and dynamic. Defects in RTK pathways have been linked to various cancers including neuroblastoma.



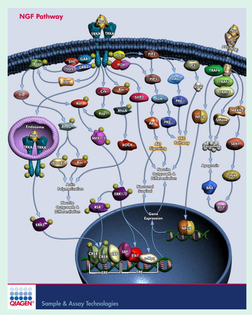


Figure 2. Detailed diagrams illustrating the complexity of NGF and GDNF signalling pathways from: http://www.qiagen.com/products/genes%20and%20pathways/pathway%20details.aspx?pwid=197

- While identifying each pathway involved in differentiation is important, it is as important to understand how the pathways interact.
- Here, I focused on two RTKs that cause differentiation in neuroblastoma cells: TrkA and Ret. TrkA and Ret were activated with their respective ligands, NGF and GDNF, both individually and together, to analyze the effects of co-stimulation.

IMMEDIATE GOAL: To improve the current understanding of interactions between these two RTK pathways. Do they act synergistically?

ULTIMATE GOAL: To contribute to a better understanding of cell differentiation and how it goes awry in neuroblastoma.

Acknowledgments

First and foremost, I would like to thank Dr. Mark Grimes for welcoming me into his lab, facilitating this project and offering guidance and support throughout.

I would also like to extend a big thanks to Juan Palacios Moreno, the graduate student in the Grimes' Lab who made himself available to answer questions, offer suggestions, and even finish procedures for me whenever I had a time conflict. It was also he who quantified the results for my western blot experiment.

Materials and Methods

Counting Experiments:

- Cells were subjected to 2nM treatments of NGF, GDNF, or NGF and GDNF along with their feedings 3 times/week.

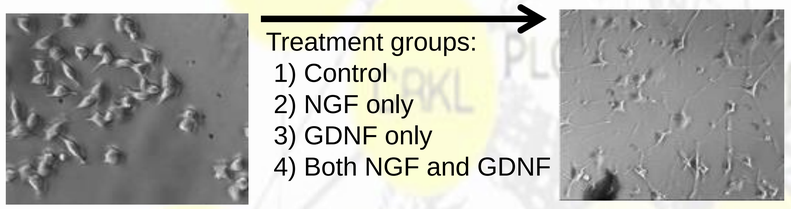


Figure 3. Illustration of experimental design. Images from: http://www.rsc.org/ej/AY/2011/c0ay00769b/c0ay00769b-f1.gif

- Counted cells growing neurites out of the total number of cells in the field of view under the microscope. Three regions were counted per well and three wells of each treatment were compiled.
  - SY5Y cells were counted after 3 weeks and again at 4 weeks.
  - LAN6 cells were counted after only 2 weeks due to the collagen coating having facilitated neurite outgrowth by providing a sturdy substrate.
- The LAN6 cells were counted again after 3 weeks of treatment to quantify the observation that cells in the control and GDNF treatment group were not adhering as well to the substrate and even appeared to be retracting neurites and 'rounding up,' or progressing towards apoptosis. These results are depicted in **Figure 6**.

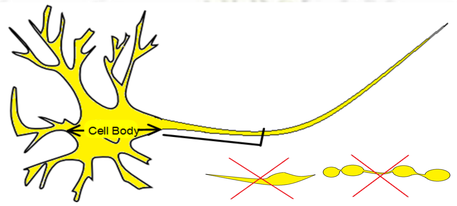


Figure 4. Samples illustrating how "neurite" was defined. For these purposes, a neurite must be greater than 1 cell body length.

Western Blots:

All treated LAN6 cells were subsequently harvested, cracked and cell contents were separated by gel electrophoresis. Blots were probed with primary antibody for phosphorylated Erk (pErk, a downstream effector of cell differentiation), and made to luminesce. Signals were captured and quantified using Image Guage V4.22 software according to signal intensity. Spillage was accounted for by summation. Western Blot results shown in **Figure 7**.

Results

In SY5Y cells:

- Combined treatment depressed neurite outgrowth in the first 3 weeks
- By the 4th week, neurite outgrowth was amplified by combined treatment, indicating that integration of signals is synergistic

In LAN6 cells:

- Neurite outgrowth was intermediate when both Ret and TrkA were stimulated. Findings thus far are summarized in **Figure 5.**
- Neurites regress without activation of TrkA by NGF, suggesting that NGF stimulation may be an absolute requirement for cell differentiation. See **Figure 6.**
- There is 10X less activated Erk (pErk) in co-stimulated cells than control. See **Figure 7.**

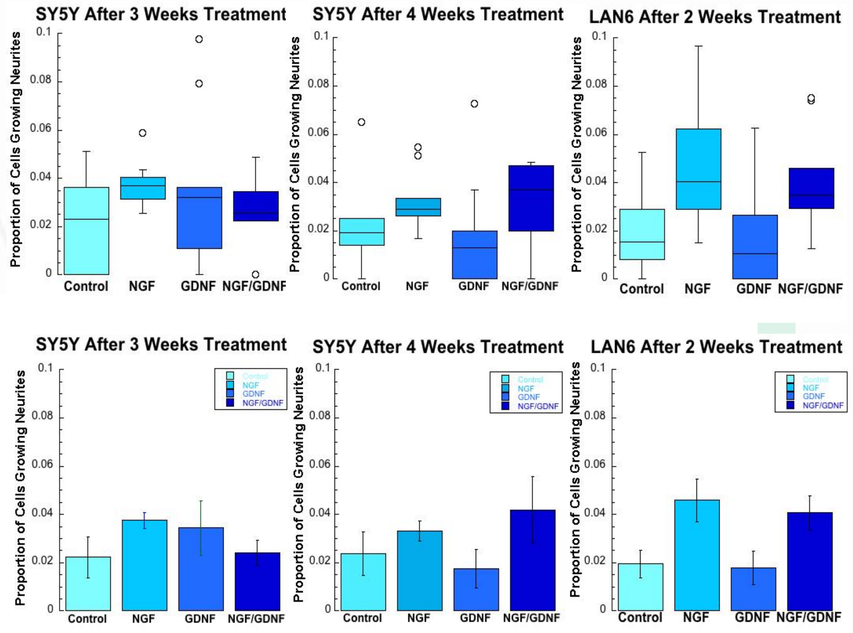


Figure 5. Proportion of SY5Y cells (after 3 and 4 weeks) and LAN6 cells (after 2 weeks) growing neurites under each of the treatment conditions presented in both box plots and bar graphs.

Figure 6. Numbers of LAN6 cells (after 3 weeks) failing to integrate with the substrate and appearing to be retracting neurites under each of the treatment conditions.

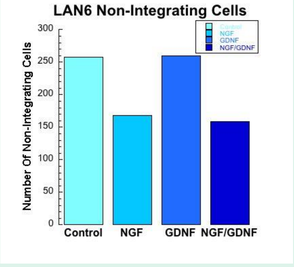
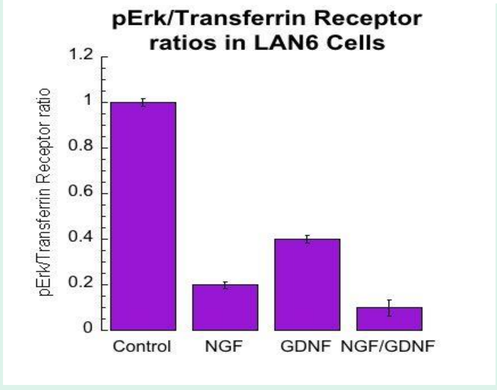


Figure 7. Western blot of LAN6 cells and pErk signal averages.



Discussion and Conclusions

The hypothesis that the Ret and TrkA pathways act synergistically was partially supported by the data; only SY5Y cells after 4 weeks of treatment displayed a synergistic response. The relationship appears to be specific to cell type and sensitive to culture substrate and treatment duration.