Stepwise Simulation Provides Insights Into NGF Signaling Pathway

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NGF is a well-known stimulus which is crucial for maintenance, survival and growth of sympathetic and sensory neurons. NGF binds to high-affinity tyrosine kinase receptor TrkA on the membrane and induces the activation of PT3 kinase, Ras, ERK and finally yields cell differentiation. Recent Studies are striving for elucidate the unknown mechanism of EGF and NGF dependent ERK signaling networks. Both transient and sustained ERK activation have already been observed in both silico and vitro experiments. However, many specific details of ERK activation remain unknown. In former research, 5 min stepwise NGF dependent ERK activation experiment has been conducted and reported in Sasagawa's paper and shown that when NGF concentration is decrease to 1ng/ml, final intensity of ERK signal will decrease significantly, whereas in our 15 min stimulation experiments, statistically, discrepancy due to different concentration NGF treatment is indiscernible.

INTRODUCTION

NGF (nerve growth factor) takes essential responsibility for survival and development of sympathetic and sensory neurons. Without the existence of NGF, the neurons will undergo cell apoptosis. Since Rita Levi-Montalcini and Stanley Cohen first discovered NGF in the 1950s, tremendous efforts have been put on NGF research. High resolution X-ray Crystal Structure of NGF was solved in 1991 and the result was published in *Nature* by McDonald et al. Recent years, as the development of system biology, people gain more insights on not merely NGF, but also the whole NGF pathway which includes NGF-dependent ERK activation, the process which regulates and determines cell differentiation.

For medical significance, NGF treatment to NGF covalent pathway has the potential to help cure several diseases of the nervous system and the application to clinical trial has already proved to be successful. Indeed, NGF and NGF signaling pathway are worthy to be underlined and attract more scientific interests.

Current studies have offered practical molecular frameworks of NGF-dependent ERK signaling pathway. Generally speaking, NGF leads to both transient ERK activation and sustained ERK activation through different protein phosphorylation cascades. NGF induces tyrosine phosphorylation of one of the subunit of NGFR (NGF receptor). Transient ERK activation by NGF depends on Ras and sustained ERK activation involves slow and sustained activation of Rap1, which is mediated by sustained TrkA activation. Then activated Rap1 activates B-Raf, followed by activation of MEK, resulting in sustained ERK activation. In preceding research, both experiments and simulation results indicates that transient ERK induced by NGF depends on the rapid temporal rates of growth factors but not on those final concentrations, while sustained ERK activation depends on the final concentration of the stimulus but not on its temporal rate of increase or decrease (S.Sasagawa et al, 2005) Based on these conclusions, we gain more insights on the physical properties of those transient and sustained ERK activation process by conducting stepwise NGF stimulation in vitro by using PC12 cells.

RESULTS

Since NGF pathway shares some of cascades with EGF (Epidermal Growth Factor) pathway, and both NGF and EGF can generate transient ERK peak (see figure 1), researches usually compare the results of EGF pathway and NGF pathway to yields better understanding of how the difference in pathway topology generates distinct ERK activation pattern.

When studying the difference between EGF-dependent ERK activation and NGF-dependent ERK activation, researches usually integrates those signaling cascades to amplify the most crucial characters of these two pathway topologies. On EGF stimulation, the network pathway presents only negative feedback from ERK,

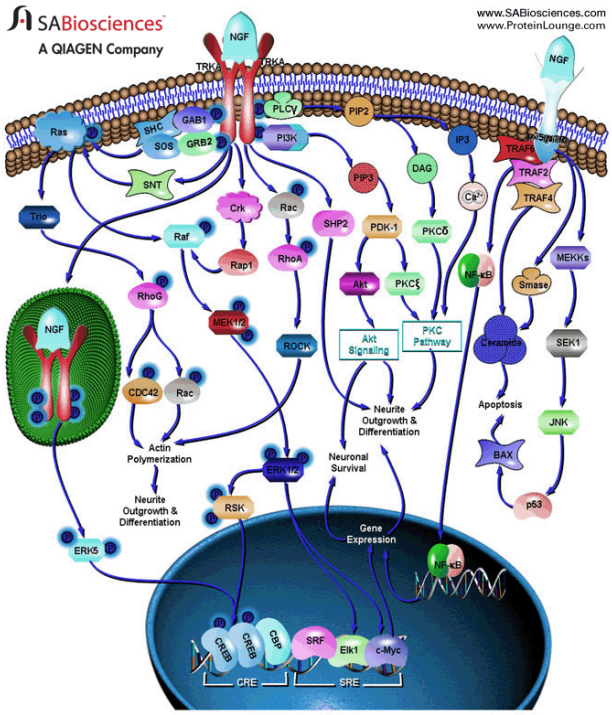

Figure 1

Figure 1 is a cartoon topology graph of NGF pathway, from which we can view the whole NGF pathway comprehensively. The major part we discuss in this paper is within the red circle.

When reaching equilibrium, we found the final concentration of phosphorylated ERK converges to a constant. Then, In Sasagawa's paper, 5 min NGF stimulation has shown that this constant is in response to the final concentration of NGF (see figure 3). From the result in silico Sasagawa offered, we can find the final concentration of ERK when the cell treated with 5ng/ml is significantly different with the concentration of ERK when treated with 1ng/ml or 10ng/ml (the saturated concentration limit). Things turn very incredible when cells are stimulated in 15min. We conducted our experiment of 15min stimulation and present the result in figure 4. When treated 5ng/ml in the beginning and do stepwise stimulation at 15min time point in 5ng/ml(control group), 1ng/ml(decrease group), 10ng/ml(increase group), the final concentration of ERK induced by different stepwise concentration remains similar, especially, 5ng/ml and 10ng/ml group.

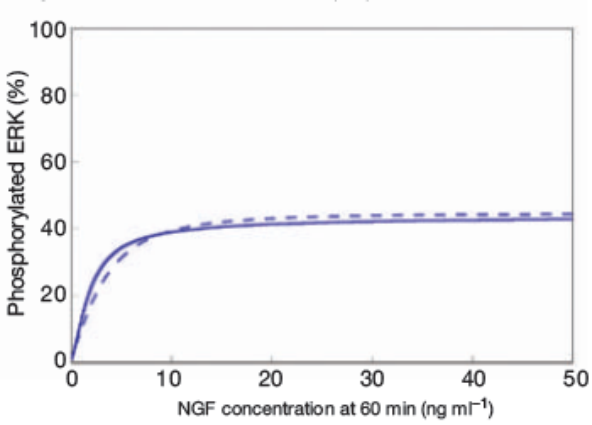

Figure 2.The in silico sustained ERK phosphorylation at 60min. Solid and dashed lines indicate ERK phosphorylation at 60 min with constant and increasing NGF stimuli, respectively. The increasing stimuli are represented as the NGF concentrations at 60 min.

From the knowledge provided by figure 2, we know TrkA as a receptor of NGF can be successfully recycled and respond to NGF changes (known by Sasagawa's paper). Therefore, cascades start from pTrkA will not suffer significant changes from 5min to 15min. However, the bi-stable ERK activation is generated by ERK activation gradually. It is obvious that the correlation of each molecule in the bi-stable pathway is gradually built due to time course. This may affects the robustness of bi-stable ERK activation and leads to the 15min stimulation results. We then focus on the bi-stable ERK activation in the view of time course.

NGF serves as a key to open up sustained ERK activation

Extracellular signaling seem hardly affects the final concentration of activated-ERK. More cell stimulation experiments should be conducted, for instance, 10min and 20min stepwise stimulation, to give more evidence to this hypothesis. We should take both final concentration and time course into our horizon to see their integrated influence to the final results. If future experiments providesevidence for the hypothesis we posted in this paper, NGF will definitely functionalize as a temporary key to open the sustained activation of ERK and determine the cell to go cell differentiation and keep survival. 5min stimulation shows differences of ERK concentration when given different concentration treatment between 0 to 10ng/ml

DISCUSSIONS

When delving into this issue deeper, molecular and structural details needed to be considered. Simultaneously, systematic view also provides perspectives to understand the mechanism of protein and protein interactions. In further research, quantitative measurement of free energy changes of the reaction in time course should be conducted and may offer more evidence as well.

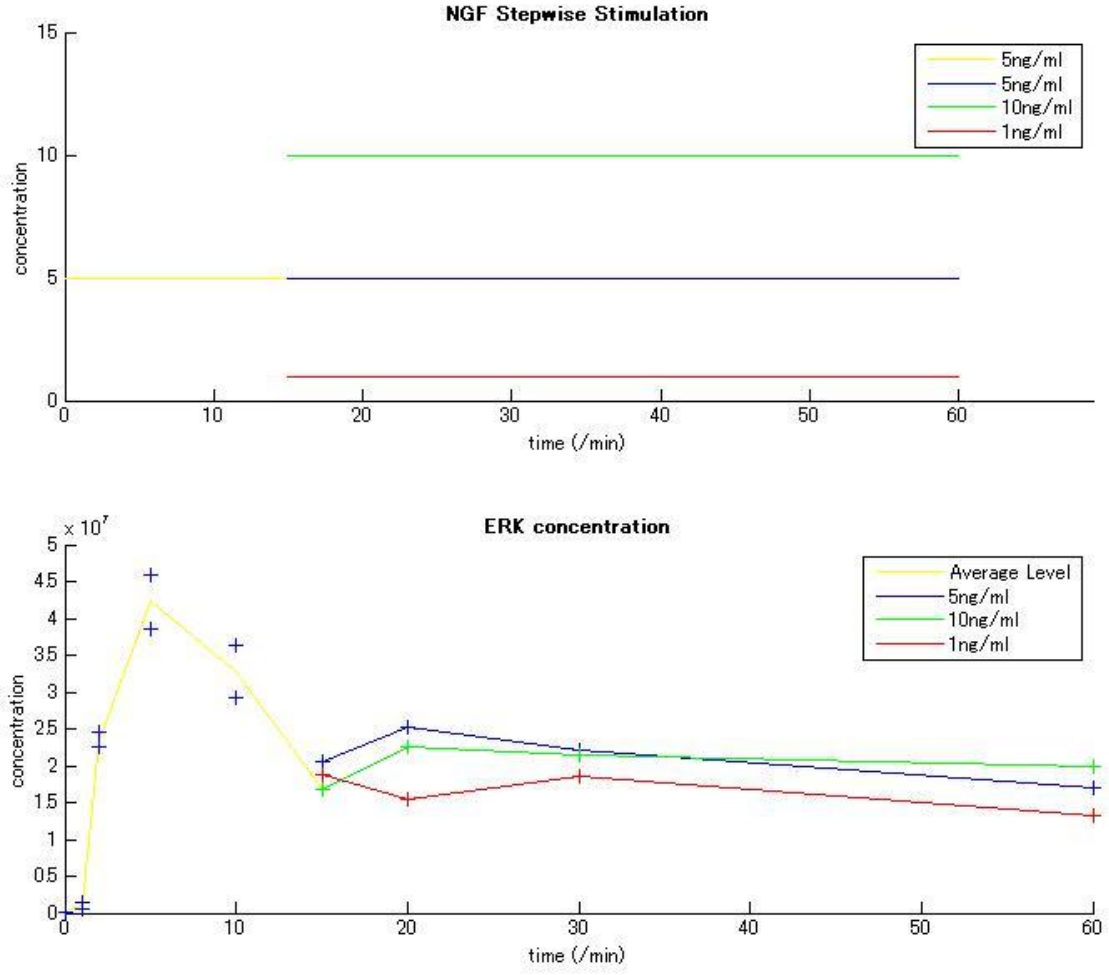

figure 3

figure 3. Stepwise increase and decrease stimulation s results. 5ng/ml and 10ng/ml NGF induced ERK activation are indistinguishable and 1ng/ml and 5ng/ml NGF induced ERK activation is insignificantly distinguishable. And in the first five min, the transient peak has occurred and plotted. The x axis is time course from 0 to 60 and the y axis represents the intensity of pro-ERK.

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METHODS

Numerical simulation of biochemical reactions and plotting.

Biochemical reactions presented in this paper involve simplified protein-protein interaction and enzyme catalytic reactions. Differential Equation Model is designed by Matlab System Biology Toolbox. Graphs are plotted by Matlab software.

Cell Culture and Starvation

PC12 cells were cultured at a density of 8×10^5 cells per 6cm dish before serum starvation. We changed the 2ml original media to the 2ml media without serum by using a electronic pipette. Then, incubate all the dishes for 16 hours at 37 degree in a CO_2 incubator.

Cell Stimulation and harvesting

First add 20ml EGF at the onset time to each concentration labeled group (5ng/ml, 10ng/ml, 1ng/ml). Then add 500ng/ml at a proper volume to 10ng/ml labeled group at the 15mins time point. (For 1ng/ml labeled group, we change the media which contains 1ng/ml concentration EGF already). Finally, Add 400 ml Lysis Buffer to harvest proteins in the cell.

We Transfered each dishes totally to sample tubes(already labeled) and centrifuged all tubes at 15000rpm at 4 degree, then sonicated all tubes for about 1 mins, centrifuged the tubes another time and stored all tubes in a refrigerator till immunoblotting.

Immunoblotting

We prepared a wide gel with 24 wells. We filled each well with proper volume of samples at a given order. Electrophoresis process was done for separating each protein on the separation gel at 35mA current for about 1 hour. Then we transferred the protein to a nitrocellulose membrane by transfer buffer for 3 hours. Then the membrane was treated by anti-phospho TrkA antibody and anti-phospho ERK0.5 antibody for overnight. The concentration of this primary antibody treatment is 1:1000. Then Secondary antibody treatment was processed with HRP-conjugated secondary antibody at a concentration of 1:5000. Finally, an enhanced chemiluminescence detection kit was used for bands detections.

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