

# Electrical stimulation promotes nerve growth factor-induced neurite outgrowth and signaling

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## Abstract

**Background** : Neurotrophins are important regulators for neural development and regeneration. Nerve growth factor (NGF) therapy has been tested in various models of neural injury and degeneration. However, whether NGF can reach target tissues and maintain effective concentration for a certain period of time remains uncertain. To facilitate neural regeneration, we investigate the possibility of combining NGF and electrical stimulation (ES) in promoting neurite outgrowth, an essential process during neural regeneration.

**Methods** : PC12 cells were seeded on collagen and indium tin oxide (ITO)-coated area on the transparent conductive devices. Cells were then subjected to the combination of ES and NGF treatment. Neurite outgrowth was compared.

**Results** : Our findings suggest that ES of 100 mV/mm together with NGF provides optimal effect on neurite outgrowth of PC12 cells. ES increases NGF-induced neurite length but reduces neurite branching, indicative of its primary effect on neurite elongation instead of initiation. One mechanism that ES enhances neurite outgrowth is through increasing NGF-induced phosphorylation of ERK1/2 (pERK1/2) and expression of Egr1 gene. ES has previously been demonstrated to increase the activity of protein kinase C (PKC). Our result indicates that activating PKC further increases NGF-induced pERK1/2 and thus neurite outgrowth.

**Conclusion** : It is likely that ES promotes NGF-induced neurite outgrowth through modulating the activity of ERK1/2.

**General significance** : Findings from this study suggest that combining ES and NGF provides a promising strategy for promoting neurite outgrowth.

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# 1. Introduction

In response to nerve injury, a series of cellular events are induced, including signaling from damaged site, recruitment of macrophage, increased synthesis of neurotrophins and regeneration of nerve fibers. To regenerate damaged or degenerating neurons, inducing neuronal differentiation and maintaining neuronal survival are essential processes. During neuronal differentiation, neurite outgrowth is an early and essential process. Various pharmacological treatments have been used to induce neurite outgrowth — one of these is the use of neurotrophins [1-5]. Neurotrophins are a family of closely related proteins that regulate many aspects of development, survival, maintenance and function of neurons. For a subset of neurons, including sympathetic neurons, nerve growth factor (NGF) is the main neurotrophin that induces neurite outgrowth and is required for the maintenance of neuronal phenotype. Upon binding to its cell surface receptor TrkA, NGF–TrkA complexes initiate the transmission of extracellular cues to intracellular signaling pathways and eventually turn on genes required for neuronal differentiation. These signaling pathways include activation of mitogen-activated protein kinase (MAPK) cascades, phosphatidylinositol-3-kinase (PI3K) stimulation of AKT and phospholipase C  $\gamma$  (PLC $\gamma$ )-dependent generation of inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) resulting in mobilization of Ca<sup>2+</sup> stores and activation of Ca<sup>2+</sup>-responsive proteins. Extracellular signal-regulated kinase 1/2 (ERK1/2) belongs to MAPK family [6-10]. In response to NGF stimulation, ERK1/2 is phosphorylated by MEK (MAPK kinase) leading to induction of genes responsible for various cellular functions, including cell proliferation, differentiation and migration. Meanwhile, NGF-induced phosphorylation of PI3K results in increased AKT activity which has been well-documented to affect neuronal survival. More recently, AKT has also been regarded as an important mediator of several aspects of neurite outgrowth, including elongation, branching and caliber. Based on the positive effects of NGF on neurite outgrowth and survival, various animal studies and clinical trials of NGF therapy for nerve injury and neurodegenerative diseases have been initiated [11-14].

Another venue to promote neurite outgrowth is through electrical stimulation (ES), which also represents a less-invasive or non-invasive approach. A number of in vivo and in vitro studies have shown promise of ES in neurite extension and regeneration of transected nerve ends [15-19]. Although the exact mechanisms by which ES enhances nerve regeneration are not well understood, some cellular responses induced by ES have been reported. These responses include neurite outgrowth, induction of neuronal tubulin, neurofilament 200, and *c-fos* gene expression [16, 20]. Activation of protein kinase C (PKC) and Ca<sup>2+</sup> mobilization are shown to be early response by ES [21-29]. However, it remains to be determined exactly what signaling pathways are initiated and are responsible for neurite outgrowth by ES.

While most of the data support a positive role of NGF in neurite outgrowth, the concentration gradient of applied NGF may exert adverse effect on other cell types. Moreover, local NGF treatment is not optimal for long-range regeneration of damaged nerve and long period of treatment using NGF raises an issue of medical cost [3-5,30-33]. Thus, investigating combination of NGF therapy with other approach to promote neurite outgrowth is of great interest. In this study, we examine whether ES would corroborate with NGF to promote neurite outgrowth of PC12 cells. We also determine whether different strengths of ES would affect NGF-induced neurite initiation, elongation and branching using PC12 cells as a model system. PC12 cell line is established as a noradrenergic clonal cell line that can be differentiated into sympathetic neurons in response to NGF [34]. We further examine the

possibility that ES-activated pathways may converge with NGF-induced signaling pathways to promote neurite outgrowth.

## 2. Material and methods

### 2.1. Reagents

Anti-ERK1/2 and bovine serum albumin (BSA), phorbol 12-myristate 13 acetate (PMA) and chelerythrine were purchased from Sigma (S. Louis, MO). Anti-pERK1/2(Thr202, Tyr204), anti-AKT, anti-pAKT(S473), anti-pSTAT3(S727), and anti-STAT3 were purchased from form Cell Signaling (Danvers, MA). Anti-Egr1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye800W-labeled anti-rabbit secondary antibody was from LI-COR Biosciences (Lincoln, NE). Alexa Fluor 700 goat anti-mouse secondary antibody, Alexa Fluor 488 secondary antibody, and rhodamine phalloidin were from Invitrogen (Carlsbad, CA). NGF and rat-tail collagen I were purchased from BD Bioscience (Bedford, MA). Anti-neuronal tubulin (Tuj1) antibody was purchased from Covance (Richmond, CA). Protein Assay Kit (Cat# PAK500) was purchased from Strong Biotech Corporation, Taiwan. Paraformaldehyde (PFA) 16% was purchased from Electron Microscopy Sciences.

### 2.2. Cell culture

The PC12 cells were purchased from American Type Culture Collection. PC12 cells were seeded on the collagen-coated dish (coated with 0.1 mg/ml rat-tail collagen in 0.02 N acetic acid) and maintained in complete medium, DMEM supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 1% l-glutamine (L-Gln) and 1% antibiotic-antimycotic (AA) (Invitrogen) under 10% CO<sub>2</sub> at 37 °C condition. Before treatment of NGF or ES, cells were deprived in serum-free media for 8 to 16 h. The serum-free media was the DMEM supplemented with 1% BSA, 1% L-Gln and 1% AA.

### 2.3. Electrical stimulation and microscopy

ES was performed by using transparent conductive devices designed and manufactured in Department of Electrical Engineering, National Tsing Hua University. The electrodes were fabricated on borosilicate glass to facilitate observation with an inverted microscope. Indium tin oxide (ITO) of 0.1 μm in thickness was deposited by radio frequency (RF) sputtering and patterned by the lift-off technique to form electrodes separated by 2 mm. Metal wires were then glued to the pads by silver paste. The bio-compatible polydimethylsiloxane (PDMS) was used to attach the glass chip in a 3.5-cm Petri dish and reinforce the wire connection. PDMS was used because of its low toxicity to cells. To evaluate neurite outgrowth, various amplitudes of ES were applied to cells via a function generator (Agilent Technologies, Taipei, Taiwan).

PC12 cells were seeded on collagen and ITO-coated area on the chips and maintained in complete medium 24 h. After 24 h, cells were deprived in serum-free medium for overnight. PC12 cells were subjected to sinusoidal stimulation potential of 100 mV, 200 mV or 400 mV alternating current, peak-to-peak, with a frequency of 100 Hz for 2 h, followed by

the addition of NGF. The distance of two electrodes was 2 mm, so the electric field generated was 50, 100 or 200 mV/mm. Images were taken using Carl Zeiss Observer Z1 microscope. Neurite length of PC12 cells was measured using ImageJ software. The longest neurite of each cell was compared. The definition of attachment point is the number of neurites connected to the soma and the endpoint is the number of the neurite tips.

#### 2.4. Immunoblotting

Cell lysates were collected in RIPA buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 150 mM NaCl, 2 mM EGTA) containing with 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethanesulphonyl fluoride (PMSF), 10 ng/ml aprotinin and 10 ng/ml leupeptin. Protein concentration of each sample was determined by protein assay kit using Bradford method. The samples with equal amount of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the indicated antibodies. The immunoblots were subsequently detected using IRDye-conjugated IgG and the Odyssey Infrared Imaging System.

#### 2.5. Immunofluorescence staining

Cells were treated without or with ES and fixed by 4% paraformaldehyde, permeabilized by 0.1% of Triton X-100, and incubated with anti-neuronal tubulin (Tuj1) antibodies, followed by incubation with the Alexa Fluor 488 secondary antibody, rhodamine phalloidin (for actin visualization) and DAPI (for visualizing the nucleus). Images were taken using Carl Zeiss Observer Z1 microscope.

#### 2.6. Statistical analysis

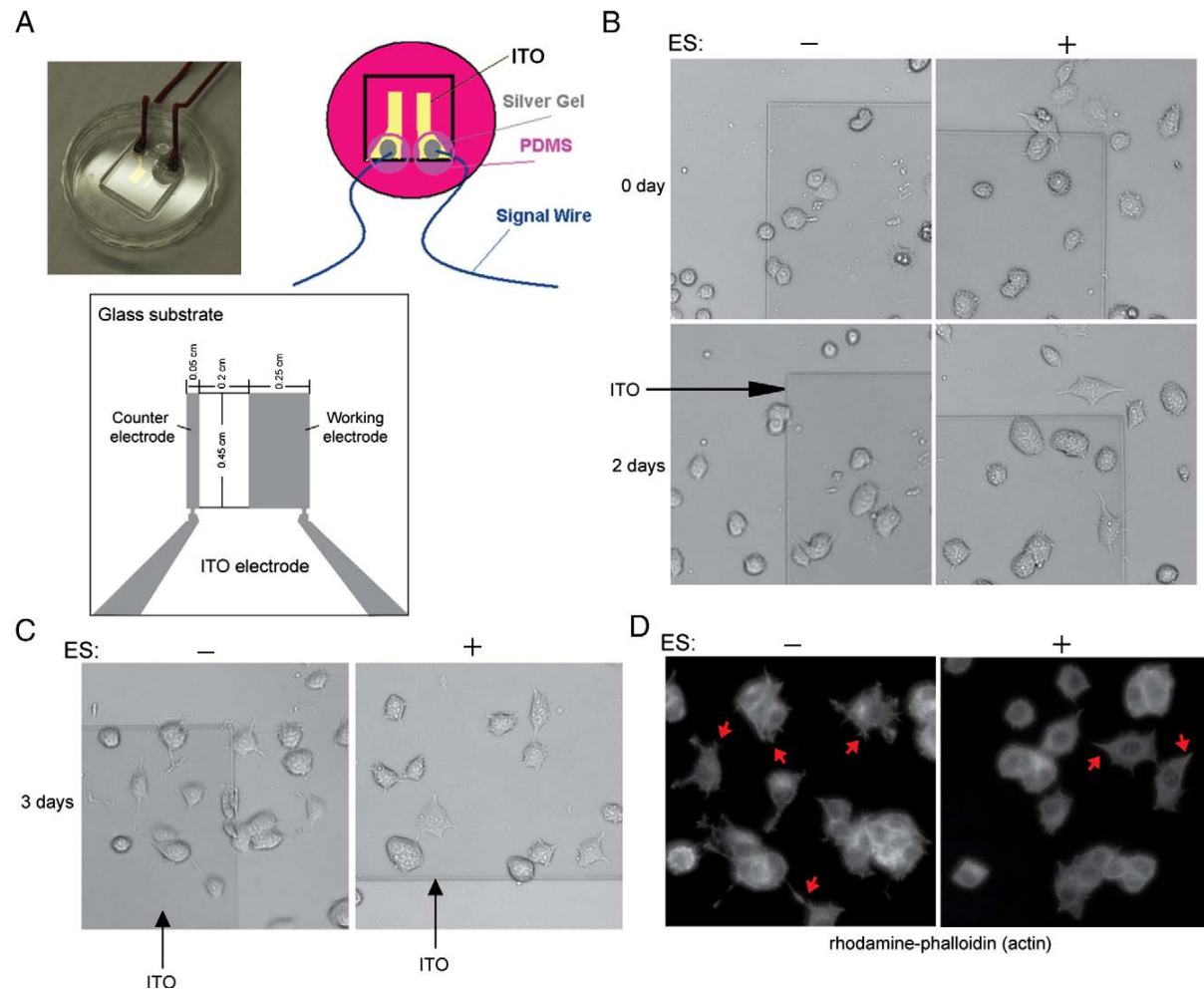
The statistical analysis of differentiation percentage, attachment points, endpoints and immunoblotting quantification were performed using one-way ANOVA. Significance (\*) is defined as  $P < 0.05$ .

## 3. Results and discussion

### 3.1. Electrical stimulation is not sufficient to induce neurite outgrowth

ES has previously been suggested to induce cellular protrusion/process or neurite outgrowth of PC12 cells[20,35]. We re-visited the effect of ES on neurite outgrowth. To this end, we have designed a transparent indium tin oxide (ITO) conductive device with electrodes of 2 mm apart on a borosilicate glass placing in culture dishes (see Materials and methods) for growing cells and imaging live cells using inverted microscope (Fig. 1A). ITO was used because of its electrical conductivity and it can be deposited as a thin film for convenient electrode design. To determine whether ES could induce neurite outgrowth of PC12 cells, PC12 cells were seeded on collaged-coated ITO chip dishes then treated with or without ES of 100 mV/mm for 2–3 days (Fig. 1B–C). As shown in Fig. 1B, ES alone was not sufficient to induce neurite outgrowth of PC12 cells. Morphologically, some cells had more spikes than others, but these cellular protrusions/processes/filopodia are not necessary

neurites. To form stable neurites, the length of neurites needs to be at least the diameter of cell body. Morphological spikes of less than one cell body (small filopodia-like protrusion of cellular process) cannot be considered as neurites. Nonetheless, actin-rich filopodia are known to precede the formation of neurites [36,37]. Using rhodamine phalloidin staining to label actin, filopodia were identified. As shown in Fig. 1D, we did not find ES of 100 mV/mm affect filopodia or neurite outgrowth of PC12 cells.



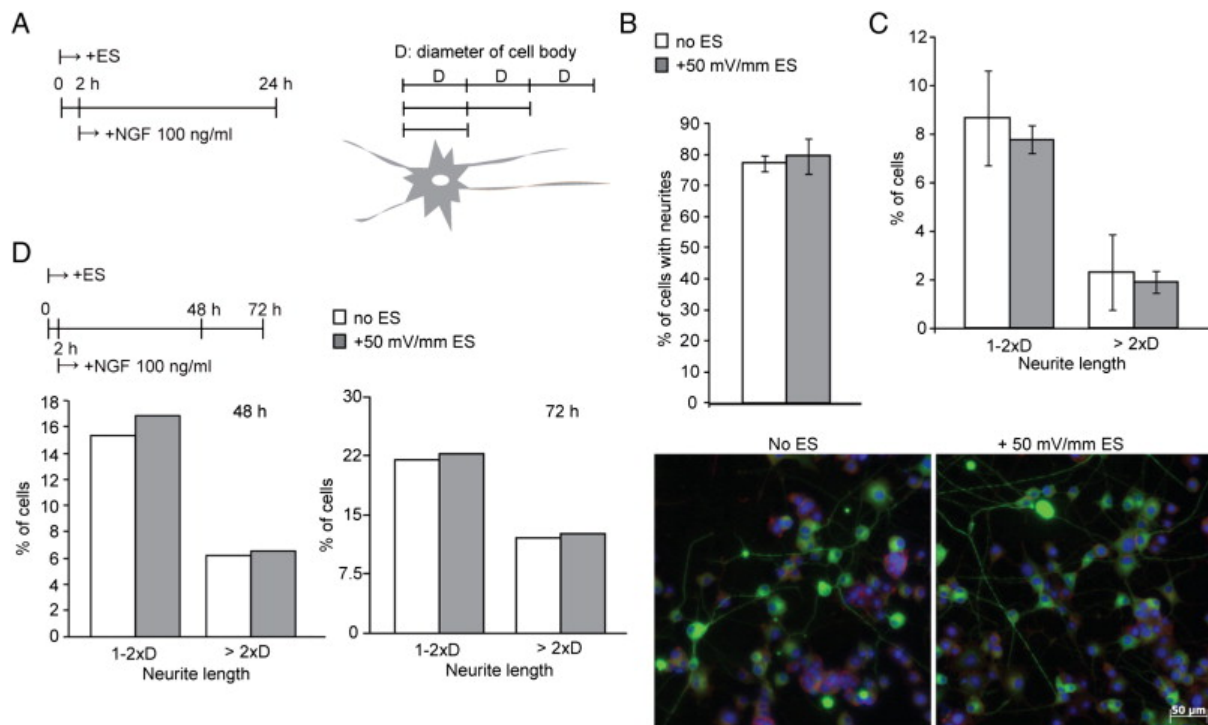
**Fig. 1.** Electrical stimulation alone is not sufficient to induce neurite outgrowth of PC12 cells. (A) Diagram of designed ITO chip for ES. Indium tin oxide (ITO) electrodes of 0.1  $\mu\text{m}$  in thickness were separated by 2 mm and coated on glass. ES was performed by using transparent electrodes fabricated on glass substrates to facilitate observation with an inverted microscope. Cells were cultured on ITO-coated area. (B) PC12 cells were treated without (-) or with (+) electric field of 100 mV/mm for 0–2 days. Live cell images are shown. Dark shaded area is ITO-coated. (C) PC12 cells were treated without (-) or with (+) 100 mV/mm ES for 3 days. Live cell images are shown. (D) Cells were treated as in (C), fixed and incubated with rhodamine phalloidin to visualize actin filaments. Red arrows indicate filopodia.

### 3.2. Effect of electrical stimulation enhances NGF-induced neurite outgrowth

NGF is known to induce neuronal differentiation of PC12 cells. We next determined whether ES would affect NGF-induced neurite outgrowth during differentiation of PC12 cells. From our previous experience, NGF exerts its effects very fast. To avoid effect of ES masked by NGF action, we subjected cells to ES 2 h before the addition of NGF to culture medium in



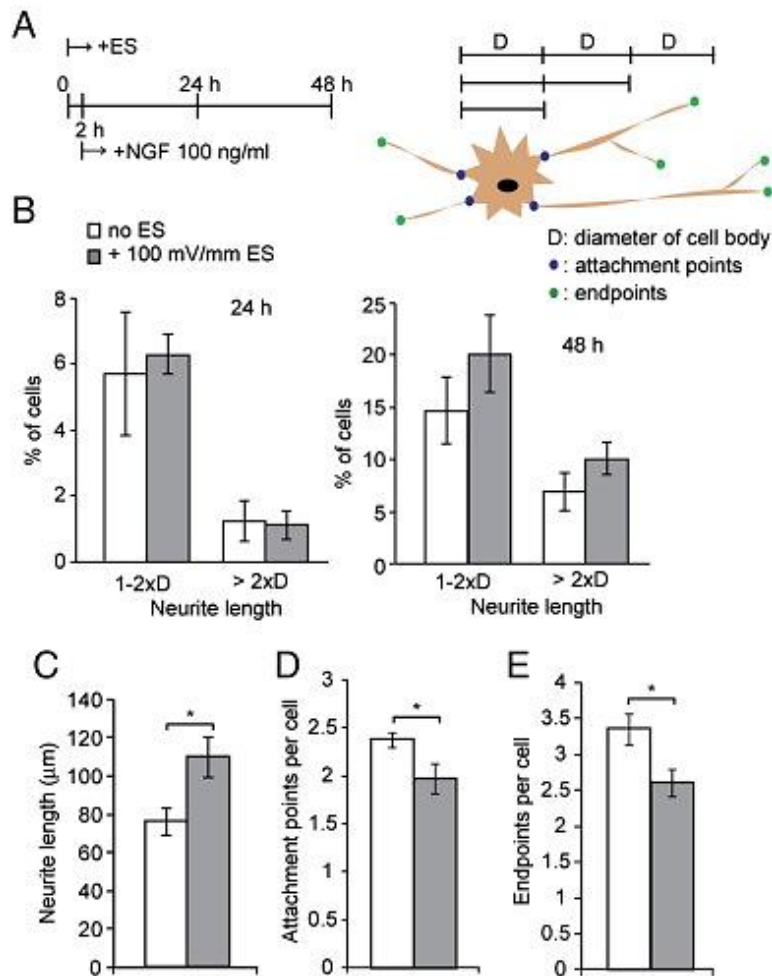
the following experiments. PC12 cells were electrically stimulated with 50 mV/mm for 2 h before adding 100 ng/ml NGF for 22 h with continuous ES. As a control, cells were treated with NGF without ES for 22 h (Fig. 2A, left panel). For measurement purpose, we define the diameter of cell body as  $D$ , and compared percentages of cells with neurites, cells with neurite length between 1 and 2 times ( $1-2xD$ ) the diameter of cell body, and cells with neurites at least twice the diameter of cell body ( $> 2xD$ ) (Fig. 2A, right panel). As shown in Fig. 2B, 50 mV/mm ES did not enhance 100 ng/ml NGF-induced neurite outgrowth. There is no difference in turns of percentages of cell population with different neurite lengths (Fig. 2C). Longer treatment of 48 or 72 h stimulation was performed with similar results (Fig. 2D). This finding suggests that 50 mV/mm ES is not capable of enhancing NGF-induced neurite outgrowth.



**Fig. 2.** 50 mV/mm electrical stimulation did not enhance NGF-induced neurite outgrowth. (A) PC12 cells were subjected to 50 mV/mm ES for 2 h before adding 100 ng/ml NGF for additional 22 h. As a control, PC12 cells were treated with 100 ng/ml NGF for 22 h. (B) Percentages of cells bearing filopodia (short protrusion or process) or neurites are shown. (C) Percentage of cells with various lengths of neurites ( $1-2xD$  or  $> 2xD$ ) is compared. 400–550 cells were counted per experiment. Values are mean  $\pm$  S.E.M. from four independent experiments. (D) PC12 cells were subjected to 50 mV/mm ES for 2 h before adding 100 ng/ml NGF. Percentage of cells with neurite length of  $1-2xD$  or  $> 2xD$  is compared. 800–900 cells were counted per experiment. Cells were fixed and incubated with rhodamine phalloidin to visualize actin filaments (red), DAPI to visualize the nucleus (blue) or anti-neuronal tubulin (Tuj1) antibody followed by Alexa Fluor 488 secondary antibody (green) for immunofluorescence staining.

We next examine whether stronger ES would enhance NGF-induced neurite outgrowth of PC12 cells. PC12 cells were stimulated with 100 mV/mm for 2 h before 100 ng/ml NGF addition and continuous ES for 22 or 46 h (Fig. 3A). NGF + ES for 22 h (Fig. 3B, left panel) did not affect the percentages of cells with neurites, whereas NGF + ES for 46 h enhanced percentages of cells with neurites (Fig. 3B, right panel). The averaged neurite length of cells treated with NGF for 46 h was 76  $\mu$ m, whereas it was 110  $\mu$ m with combined treatment of NGF and ES (Fig. 3C). To understand whether ES affects the number of primary neurites or

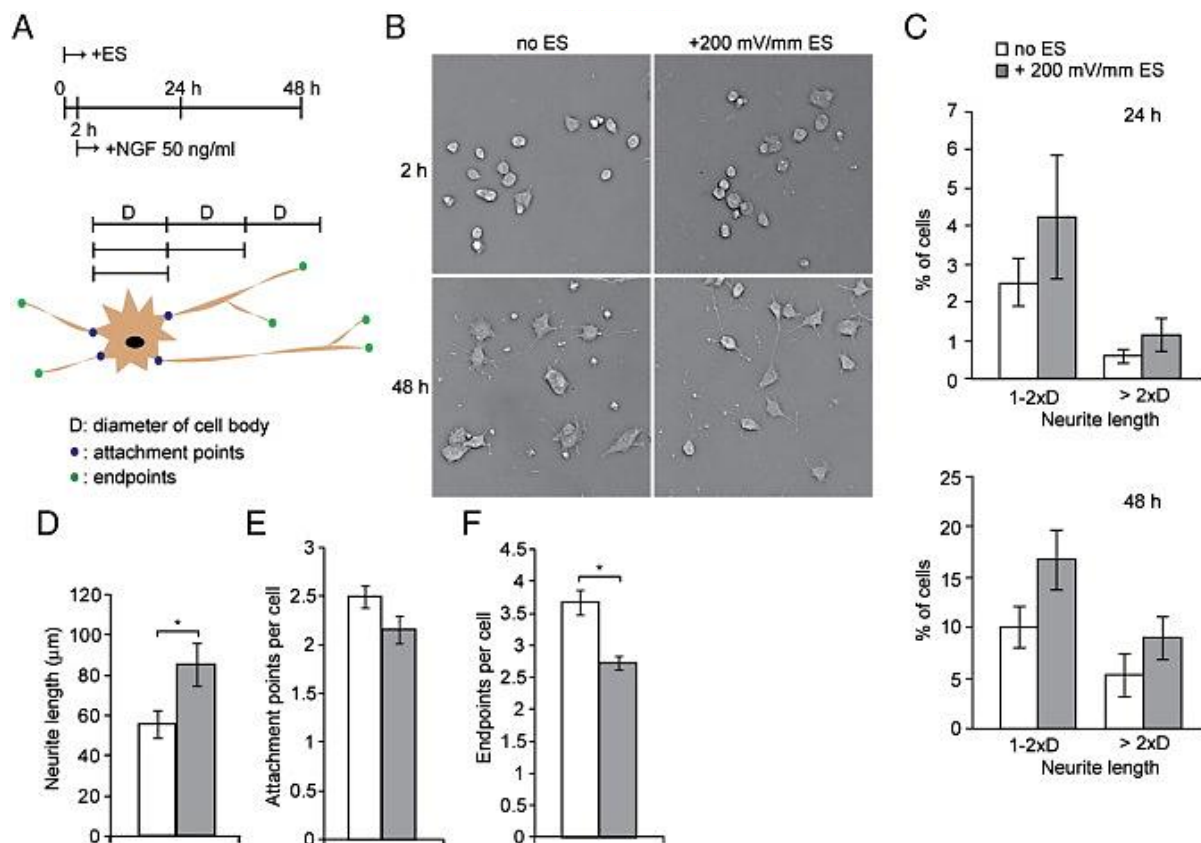
neurite branches, the numbers of attachment points and endpoints were measured and counted as depicted in the right panel of Fig. 3A. Interestingly, the numbers of attachment points were reduced 17% and endpoints were reduced 24% with 100 mV/mm ES (Fig. 3D–E). Attachment points represent the number of primary neurites extending from soma. Number of endpoints infers to the degree of neurite branching. These two parameters were consistently reduced with ES suggesting that 100 mV/mm enhances neurite elongation instead of initiation. This finding also echoes the result from Fig. 1 that ES alone is not sufficient to initiate neurites. Only when NGF induces neurite initiation, could ES enhance neurite elongation.



**Fig. 3.** 100 mV/mm electrical stimulation enhances NGF-induced neurite elongation of PC12 cells. (A) PC12 cells were subjected to 100 mV/mm ES 2 h before adding 100 ng/ml NGF for 22 or 46 h. As a control, PC12 cells were treated with 100 ng/ml NGF for 22 or 46 h. Neurite length was calculated and compared. Attachment points and endpoints were counted. (B) Percentages of cells with neurite length of 1–2xD or > 2xD in either condition were calculated. 600–900 cells were counted per experiment. Values are mean  $\pm$  S.E.M. from four independent experiments. Averaged neurite length (C), attachment points (D) and endpoints (E) per cell were measured from three independent experiments. 70–100 cells were counted per experiment. Values are mean  $\pm$  S.E.M. (\*:  $P < 0.05$ , one-way ANOVA).

One of the problems of NGF therapy in animal studies is the non-target effect of NGF. Reducing concentration of NGF may be beneficial in this regard if higher ES can compensate the trophic effect of NGF. To this end, PC12 cells were stimulated with 50, 100 or 200 mV/mm for 2 h followed by 50 ng/ml NGF treatment with continuous ES for 22 or 46 h. When cells were treated with 50 ng/ml NGF, applying either 50 or 100 mV/mm ES for 22 or 46 h did not result in significant difference of neurite outgrowth (Supplemental Fig. 1S). Combination of 50 ng/ml NGF and ES of 200 mV/mm, on the other hand, enhanced the percentages of PC12 cells with neurites (Fig. 4B). The percentage of cells with 1–2xD or > 2xD neurites was increased by ES (Fig. 4C). Averaged neurite length increased 56% with ES compared to control (Fig. 4D). The reduction of attachment points and endpoints was similar to those treated with 100 mV/mm and 100 ng/ml NGF (Figs. 4E–F and 3D–E).

Results from Fig. 3 showed that 100 mV/mm ES increased NGF-induced neurite length approximately 45% (Fig. 3). When NGF concentration was reduced to 50 ng/ml and together with 200 mV/mm stimulation, neurite length increased 56% (Fig. 4). These findings seem to suggest that increasing the strength of ES further increases neurite elongation. However, overall neurite length and percentages of cells with neurites under 200 mV/mm and 50 ng/ml NGF are not better than the condition of 100 mV/mm and 100 ng/ml NGF (Fig. 3–4). This result could potentially due to cellular toxicity caused by 200 mV/mm stimulation.



**Fig. 4.** 200 mV/mm electrical stimulation enhances NGF-induced neurite length, reduces attachment points and endpoints. (A) PC12 cells were subjected to 200 mV/mm ES for 2 h before adding 50 ng/ml NGF for 22 or 46 h. As a control, PC12 cells were treated with 50 ng/ml NGF for 22 or 46 h. (B) Representative live cell images of 2 and 48 h stimulated cells are shown. (C) Percentages of cells with neurite length of 1–2xD or > 2xD in each condition were calculated. 350–500 cells were counted per experiment. Averaged neurite length (D), attachment points (E) and endpoints (F) per cell were measured from three independent experiments. 70–100 cells were counted per experiment. Values are mean  $\pm$  S.E.M. (\*:  $P < 0.05$ , one-way ANOVA).



### 3.3. Electrical stimulation enhances NGF-induced pERK1/2 level

Several signaling pathways are implicated in NGF-induced neuronal differentiation of PC12 cells, including MEK–ERK1/2, PI3K–AKT pathways, and pathways involve activation of PLC $\gamma$ , PKC and signal transducers and activators of transcription-3 (STAT3) [38]. To determine whether ES modulates any of these pathways, PC12 cells were treated with or without ES with 50 mV/mm for 2 h followed by NGF treatment (Fig. 5A). Phosphorylations of ERK1/2, AKT and STAT3 were examined via western blotting. As shown in Fig. 5B–C, ES enhanced NGF-induced pERK1/2 level approximately 40% but did not change pSTAT3(S727) and pAKT(S473) levels, compared to those without ES. This result raises a possibility that ES may enhance NGF-induced neuronal differentiation through regulating MEK–ERK1/2 pathway. We thus examined whether different amplitudes of ES differentially affects MEK–ERK1/2 pathway. To this end, PC12 cells were stimulated with 0, 50 or 100 mV/mm for 2 h before NGF addition for 22 h. As shown in Fig. 5D, 50 mV/mm ES increased 40%, whereas 100 mV/mm ES increased 70%, of NGF-induced pERK1/2 level compared to no ES controls. Similarly, PC12 cells subjected to 200 mV/mm stimulation also increased NGF-induced pERK1/2 level compared to no ES controls (Fig. 5D, right panels). Moreover, 100 mV/mm ES enhanced NGF-induced expression of early growth response protein 1 (Egr1), a downstream effector of MEK–ERK1/2 pathway (Fig. 5E). Taken together, these results provide important insights that ES synergistically enhances NGF-induced MEK/ERK1/2–Egr1 pathway to promote neurite outgrowth.

ES has previously been suggested to regulate neurite outgrowth through regulating PKC activity [21,22]. To investigate whether activating PKC would synergistically promote NGF-induced pERK1/2, PC12 cells were pretreated with or without PKC activator, PMA, followed by the addition of NGF. As shown in Fig. 6A, activating PKC enhanced NGF-induced pERK1/2 level more than 2-fold. Consistent with a previous study showing that PKC inhibitor blocked the effect of ES but not NGF [20], our result also showed that treating cells with PKC inhibitor, chelerythrine, did not affect NGF-induced pERK1/2 (Fig. 6B). It is thus likely that ES activates PKC to enhance NGF-induced MEK–ERK1/2 signaling, gene expression and thus neurite outgrowth (Fig. 6C).

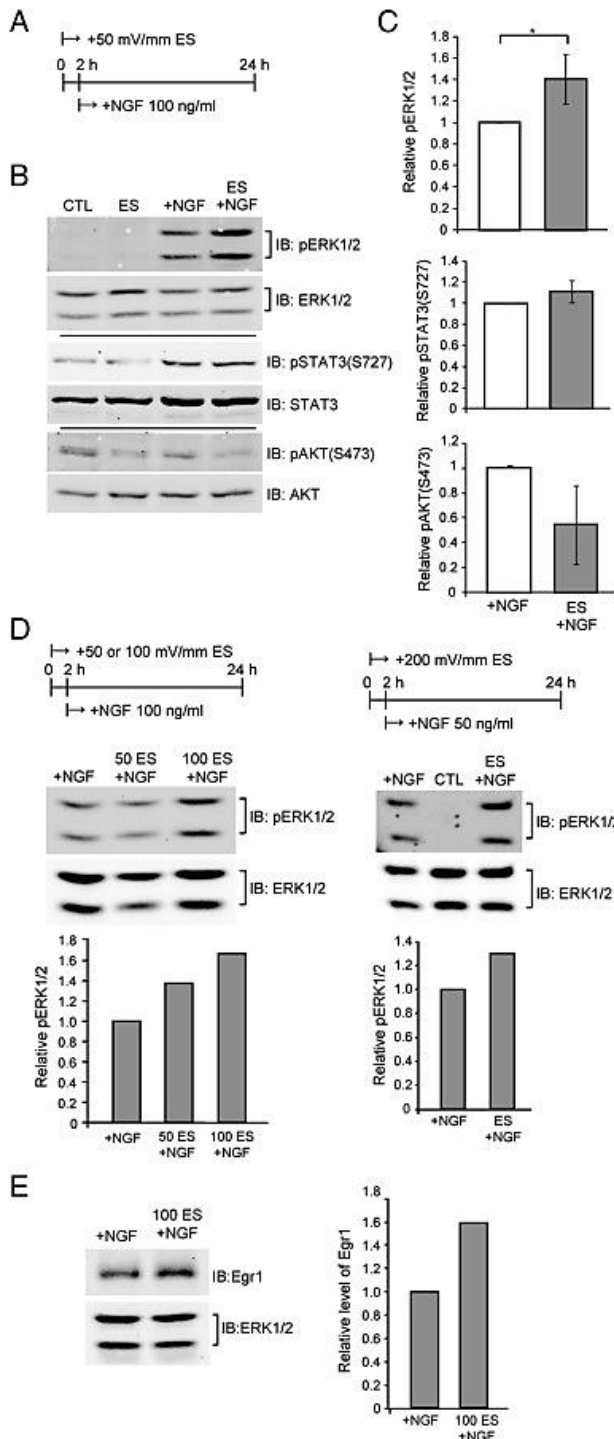
## 4. Conclusion

In this study, we explore the strategy of combining NGF and ES on promoting neurite outgrowth. Our results demonstrate that ES promotes NGF-induced neurite elongation. Among the conditions we have tested, combining 100 mV/mm ES and 100 ng/ml NGF provides the optimal effect on neurite outgrowth and percentage of cells with neurites (Fig. 3). We further demonstrate that ES enhances NGF-induced signaling and gene expression, specifically MEK–ERK1/2 pathway and Egr1 expression (Fig. 5). While ES robustly activates PKC as suggested by several studies [21,22], we find that activation of PKC leads to enhancement of NGF-induced pERK1/2. These findings provide important insights into how ES may regulate NGF-induced signaling and gene expression to promote neurite outgrowth. The protocol used in this study could potentially be considered for treatment of neurodegeneration or nerve injury.

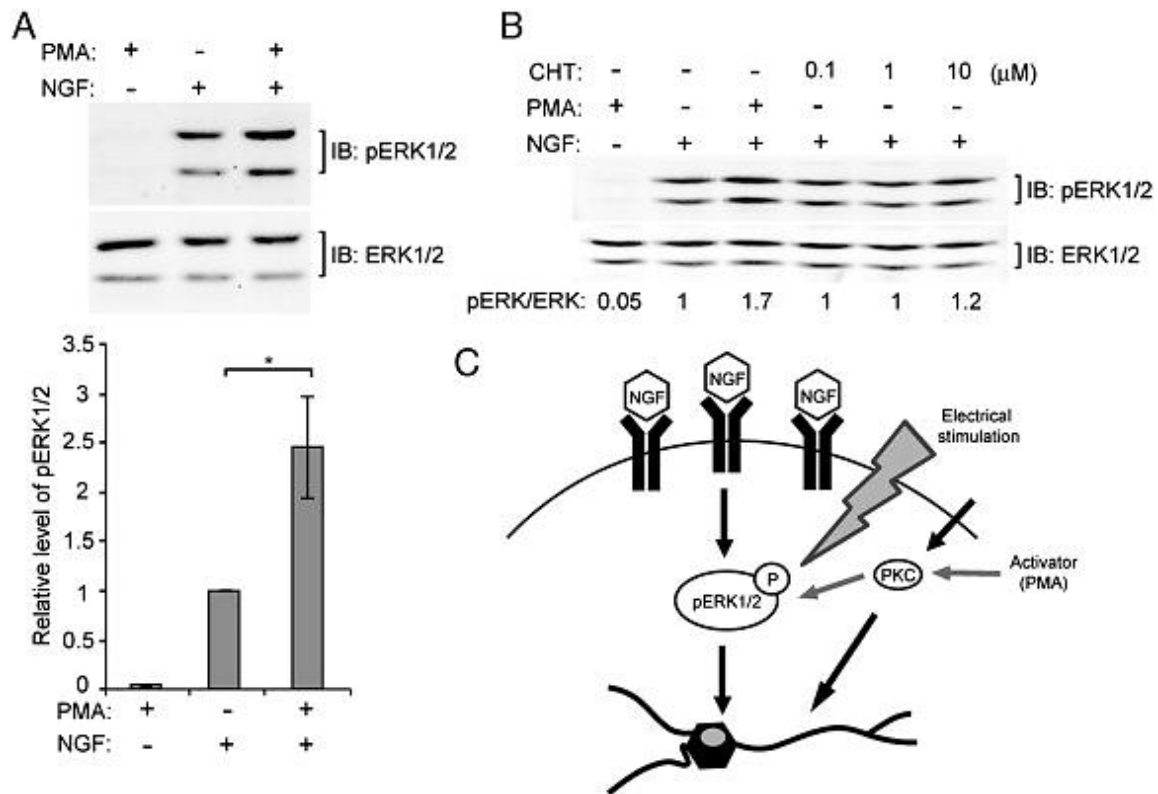
Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2013.04.007>.

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**Fig. 5.** Electrical stimulation enhances NGF-induced pERK1/2. (A) PC12 cells were subjected to 50 mV/mm ES for 2 h before adding 100 ng/ml NGF for 22 h with continuous ES. As a control, PC12 cells were treated with 100 ng/ml NGF for 22 h. (B) Cells were treated as in (A), cell lysates were collected and equal amount of proteins was separated by SDS-PAGE and immunoblotted with anti-pERK1/2, ERK1/2, pSTAT3(S727), STAT3, pAKT(S473) and AKT antibodies. Representative blots are shown from three independent experiments. (C) Relative levels of pERK1/2, pSTAT3(S727) and pAKT(S473) were normalized to total ERK1/2, STAT3, and AKT levels and compared to NGF only group (defined as 1, white bars). Values are mean ± S.E.M. from three independent experiments. (\*: P < 0.05, one-way ANOVA). (D) PC12 cells were subjected to 50 or 100 mV/mm ES for 2 h before adding 100 ng/ml NGF for 22 h with or without continuous ES. Cell lysates were collected and equal amount of proteins was separated by SDS-PAGE and immunoblotted with anti-pERK1/2 and ERK1/2 antibodies. Levels of pERK1/2 were normalized to total ERK1/2 levels and compared to NGF-only group (defined as 1). PC12 cells were subjected to 200 mV/mm ES for 2 h before adding 50 ng/ml NGF for 22 h with or without continuous ES. As a control, PC12 cells were treated with 50 ng/ml NGF for 22 h. Cell lysates were collected and equal amount of proteins was separated by SDS-PAGE and immunoblotted with anti-pERK1/2 and ERK1/2 antibodies. Levels of pERK1/2 were normalized to total ERK1/2 levels and compared to NGF-only group (defined as 1). (E) PC12 cells were subjected to 100 mV/mm ES for 2 h before adding 100 ng/ml NGF for 2 h with or without ES. Cell lysates were collected and equal amount of proteins was separated by SDS-PAGE and immunoblotted with anti-Egr1 antibody. Relative levels of Egr1 were normalized to total ERK1/2 levels and compared to NGF-only group (defined as 1).



**Fig. 6.** Activated PKC involves in NGF-induced pERK1/2. (A) PC12 cells were pre-treated with or without 162 nM PMA for 1 h, followed by 100 ng/ml NGF stimulation for 10 min. Cell lysates were collected and equal amount of proteins was separated by SDS-PAGE and immunoblotted with anti-pERK1/2 and ERK1/2 antibodies. Representative blots are shown from three independent experiments. Levels of pERK1/2 were normalized to levels of total ERK1/2 and then compared to NGF-treated group (defined as 1). Values are mean  $\pm$  S.E.M. (\*:  $P < 0.05$ , one-way ANOVA). (B) PC12 cells were pre-treated with 162 nM PMA or the indicated concentration of CHT (chelerythrine) for 1 h, followed by 100 ng/ml NGF stimulation for 10 min. Cell lysates were analyzed by western blotting as in (A). The relative level of pERK1/2 was normalized to total ERK1/2 and ratio of pERK/ERK was shown at the bottom of the blots. (C) Working model. NGF activates MAPK - ERK1/2 pathway to induce neuronal differentiation of PC12 cells. ES was previously shown to activate PKC pathway to regulate neurite outgrowth. We show that activating PKC by PMA enhances NGF-induced pERK1/2 and likely contributes to enhanced neurite outgrowth.

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