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EFFECT OF COMBINING FK506 AND NEUROTROPHINS ON NEURITE BRANCHING AND ELONGATION

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Abstract

Introduction—There is a clinical need to improve the outcomes of peripheral nerve regeneration and repair after injury. In addition to its immunosuppressive effects, FK506 (tacrolimus) has been shown to have neuroregenerative properties. To determine biologically relevant local FK506 and growth factor concentrations, we performed an *in vitro* bioassay using dorsal root ganglion (DRG) from chicken embryos.

Methods—Neurite elongation and neurite branching were analyzed microscopically after addition of FK506, glial cell line-derived neurotrophic factor (GDNF), and nerve growth factor (NGF), each alone and in combination.

Results—FK506 induced modest neurite elongation (~500–800 μm) without improving neurite branching significantly. The combination of FK506 with NGF, GDNF, or both, exerted a potentiating or competitive effect on neurite elongation (~700–1100 μm) based on dosage and competitive effect on neurite branching (~0.2–0.4).

Conclusions—These results strongly suggest that the interaction of FK506 with GDNF and NGF mediates distinct enhancement of neurite growth.

Keywords

dosage; FK506; GDNF; nerve regeneration; Neurite growth *in vitro*; NGF; potentiation

Peripheral nerve injuries have been a clinical challenge over the years.¹ The extensive network of nerve tissue throughout the body makes peripheral nerves prone to external trauma.² Peripheral nerve injuries frequently produce weakness, chronic pain, and neuropathies that lead to severe disability.³ Nerve autograft has been the gold standard for treatment of injuries that result in a nerve gap.¹ Autograft involves harvesting donor nerve tissue and implanting the graft across the nerve gap.³ Nerve autografts have been comparatively successful, but there are problems associated with donor tissue availability,² obtaining a donor nerve by means of single or multiple surgeries, multiple scarring sites, neuroma formation, and limited success at repairing gaps larger than 10 mm.² These issues

led to fabrication of hollow, tube-like nerve guiding conduits to span gaps in nerves and provide structural support for the transected nerve.⁴ These nerve guides, although structurally helpful, do not effectively replicate a nerve autograft in terms of support cells and neurotrophism and thus are not as effective at repairing large nerve gaps.⁴ Following a peripheral nerve injury, neurites will tend to regrow and cross short gaps, but they need guidance and the ability to overcome the scarring process.⁵

To improve the repair process, in addition to providing guidance, therapeutic agents need to be provided at the site of transection or injury.⁶ Several drugs and growth factors have been shown to be effective in enhancing neurite outgrowth across nerve gaps.⁶ A study of nerve growth factor (NGF)-loaded microspheres has shown potential for repair of nerve gaps.⁷ Collagen tubes loaded with NGF alone or in combination with glial cell line-derived neurotrophic factor (GDNF) have produced neurite elongation *in vitro*.⁸ Use of NGF or GDNF within a specified concentration range has resulted in improved neurite growth in chick dorsal root ganglia (DRG).⁹ Several papers suggest that improved peripheral nerve regeneration can be expected by supplying neurotrophins.^{5,10}

The data obtained from several *in vivo* studies have indicated that NGF and GDNF synthesis is also upregulated during an inflammatory process.^{11,12} After a peripheral nerve injury, proliferating and reactive Schwann cells produce growth factors, cytokines, and growth-associated proteins, which play key roles in axon regeneration and nerve repair.^{13,14} It has been observed that exogenously administered NGF and GDNF increase both the number and myelination of regenerating axons.^{15,16} This is due to effects of NGF and GDNF signaling both on regenerating nerve fibers and on Schwann cells and inflammatory cells,¹⁷ and Schwann cell migration is thought to precede and promote axon elongation into repair sites.¹⁷

FK506 was approved by the FDA in 1994 for liver transplants and is one of the main systemic immunosuppressants used to prevent nerve allograft rejection.¹⁸ It has also been observed to have neuroregenerative properties when administered after peripheral nerve injuries.^{19,20} It can enhance the activity of NGF by increasing the sensitivity of cells toward smaller concentrations of the growth factor.^{21,22} The complex of FK506 with FK506-binding protein-12 inhibits the phosphatase activity of calcineurin, resulting in accumulation of phosphorylated substrates, including nuclear factor of activated T-cells. This phosphorylated nuclear factor of activated T-cells functions as a regulator of the transcription of numerous genes, including interleukin-2 and, therefore, induces the immunosuppressive effects of FK506.²³

There is increasing evidence that the beneficial effects of FK506 on neuroregeneration are unrelated to calcineurin inhibition and thus immunosuppression.^{19,21} This first became evident as studies began to demonstrate that cyclosporine A, another immunosuppressant that works through calcineurin inhibition, does not have the same neuroregenerative properties.²³ Several research groups have demonstrated that nonimmunosuppressive FK506-binding protein ligands lack the ability to bind calcineurin but promote neurite growth *in vitro* and stimulate regeneration of peripheral nerves in rats.²⁴ Some studies have suggested that the neuro-regenerative effects of FK506 might result from interactions with

FK506-binding protein 52.²⁵ While several *in vivo* small animal studies have demonstrated improved rates of nerve regeneration with systemic FK506,^{26–30} not many detailed studies have confirmed the dosage activity profile of FK506 treatment alone or in combination with other growth factors. For this purpose, we have developed an *in vitro* assay using chicken embryonic DRGs which exhibit similarities to other animal neuronal systems.

The study we report here also assessed the regulation of protein kinase B (Akt) and phosphorylated Akt (pAkt) in the process of neurite growth after single and combined treatment with FK506 and neurotrophins. Akt is a multifunctional regulator of cell survival, growth, and glucose metabolism.^{31–33} It functions as a major downstream target of phosphatidylinositol 3-kinase (PI3-K), and the PI3-K pathway is involved in nerve growth factor-dependent neuronal survival.^{33–35} Data suggest that FK506 treatment enhances nerve growth through activation of the Ras/Raf/MAP kinase signaling pathway downstream of PI3K-Akt.²⁸ GDNF also activates the Ras/MAP kinase and PI3K/Akt pathways.³⁶ The goal of this study was to determine how neurite growth is affected by FK506, NGF, GDNF, and the combinations of FK506 with either NGF or GDNF or both in terms of neurite length and branching.

MATERIALS AND METHODS

DRG-Explant Cultures

Fertilized chicken eggs (Merrills Poultry, Paul, Idaho) were incubated at ~39 °C under 100% relative humidity for 12 days. The eggs were first cleaned with 70% ethanol and then opened to collect the embryos. DRGs were dissected from the embryos under a stereomicroscope using a standard dissection procedure.³⁷ They were separated carefully from connective tissue for culturing in 24-well plates coated with laminin (1 µg/ml). Dulbecco Modified Eagle Medium (DMEM) F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% Antimycotic/Antibiotic solution were added to each well. DRGs were plated at a density of 1 per well, and growth factors were added as specified below. Cultures were maintained in a humid atmosphere at 37 °C and 5% CO₂ for 72 h. Unless specified otherwise, all reagents for cell culture were purchased from Fisher Scientific (Pittsburgh, Pennsylvania).

Drug Dosing

For each experimental condition including control, 4 DRGs of similar size were used, and the experiments were repeated 3 to 4 times. In the single growth factor experiments, the DRGs were treated with increasing concentrations of either NGF (Sigma Aldrich, St. Louis, Missouri) (0.1, 1, 5, 10, 100 ng/ml), GDNF (R&D systems, Minneapolis, Minnesota) (0.1, 1, 5, 10, 100 ng/ml), or FK506 (Astellas Pharma, Northbrook, Illinois) (1, 5, 10, 20, 50 ng/ml). For control, DRG-explants were incubated in cell culture medium matrix (DMEM+10% FBS+1% Antimycotic) without any growth factor or drug. In the experiments using combined growth factors (NGF/GDNF, NGF/FK506, GDNF /FK506, and NGF/GDNF/ FK506), the DRGs were treated with random dosages chosen using the statistical software Minitab 17. All possible combinations for each group were subjected to a fractional factorial

design in the software, which both randomly and systematically selected dosages that were tested to predict the response of remaining combinations.³⁸

We used a fractional factorial design, because for experiments with many factors and levels, full factorial designs can lead to large amounts of data. Fractional factorial designs use a fraction of the runs required by full factorial designs. A subset of experimental treatments is selected based on an evaluation (or assumption) of which factors and interactions have the most significant effects.³⁹ The neurotrophic factor dilutions were prepared with the cell culture medium matrix (DMEM+10% FBS+1% Antimycotic).

Quantification of Neurite Outgrowth: Neurite Length and Branching

After 72 h in culture, DRGs were fixed in methanol, imaged using a widefield microscope (Nikon Spinning disk) with a phase contrast lens, and images were captured with a digital camera at 4 × magnification. Phase contrast images of DRGs were taken after 3 days of incubation, and the average neurite length and branching were measured.

Neurite length and branching measurements were done using a previously discussed procedure.⁹ The area of the ganglion body (A_{DRG}) and the total area of the DRG with the growing neurites (A_{tot}) were measured using ImageJ 1.31v software (National Institutes of Health, Bethesda, USA). Average neurite length (I_{avg}) was calculated by: $I_{\text{avg}} = (A_{\text{tot}}/\pi)^{1/2} - (A_{\text{DRG}}/\pi)^{1/2}$. Neurite branching was calculated from the area occupied by the neurites (A_{neurite}), which was determined using the threshold function (range of 100 ± 25 to 230 ± 25 pixels) available in ImageJ. Neurite branching = $A_{\text{neurite}}/(A_{\text{tot}} - A_{\text{DRG}})$

DRG Neurite Growth Rate

Images of DRGs were taken every 24 h during 3 days of incubation, and average neurite length was measured at each time point as described above. The cultured explants were not fixed for the growth rate study. From these measurements, the average neurite growth rate was determined.

DRG Lysate and Western Blot

Six DRGs were plated per well in a 6-well plate and treated with different single and combined dosages for 72 h. Cells were then lysed in Cell Extraction Buffer (Bio-source) according to the manufacturer's protocol. Cell lysates suspended in Laemmli sample buffer (Bio-Rad, Hercules, California) were heated at 95 °C for 5 min, separated by SDS-PAGE gel (Bio-Rad), and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Massachusetts). Membranes were incubated with the following primary antibodies: AKT (1:1,000, Cell Signaling Technology) and pAKT (1:2,000, Cell Signaling Technology). After washing with phosphate buffered saline (PBS) containing 0.1% Tween, membranes were incubated with a 1:5,000 dilution of horseradish peroxidase conjugated Anti-Rabbit secondary antibody (Cell Signaling Technology). Antibody was detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Massachusetts). To control for protein loading, membranes were stripped and probed with a 1:5,000 dilution of mouse anti-GAPDH (Novus Biologicals, Littleton, Colorado). Scanned autoradiographic blot images were quantified using the NIH ImageJ densitometry software.

Immunocytochemistry and Fluorescence Microscopy

DRGs were cultured for 72 h and then fixed with methanol for 10 min. Cells were washed with PBS, incubated with PBS containing Triton X-100, treated with blocking buffer containing 1% BSA (bovine serum albumin) at room temperature for 30 min, and incubated with primary antibody (β tubulin 1:200 dilution in 1% BSA in PBS-Triton X100) overnight at 4 °C. Cells were then washed and incubated for 1 h at room temperature with a secondary antibody (Rabbit 568 red 1:500 dilution in 1% BSA). Image acquisition was accomplished using Nikon Spinning disk widefield microscope and NIS Elements software.

Statistical Analysis

The data were analyzed using a multivariable linear regression model in StataIC 13. The outcome variable was neurite length and neurite branching. Specific doses of a given drug or drug combination were included in the model as indicator variables rather than assuming a specific form of dose-response, such as a linear increase across the range of doses. Interaction terms were used to test if the effects of drug combinations were greater than the sum of the effects of individual drugs. Comparisons between specific drug-dose combinations were made by varying the referent drug-dose combination in the included indicator variables, or using Wald posttest comparisons, where either approach produced comparisons analogous to independent sample *t*-tests. All reported *P*-values are for 2-sided comparisons. *P*-values ≤ 0.05 were considered significant.

In our experiments, the goal was to identify the drug(s)-dose(s) combination that provided the greatest response or to observe some range of effective doses. Statistical comparisons are provided merely descriptively to indicate where the maximum or plateau of the curve separates from the ineffective drug(s)-dose(s) combinations.

RESULTS

Neurite Elongation

Single Growth Factor—FK506 was observed to induce neurite elongation in the lower concentration range, and the average neurite length of DRGs exposed to 1 ($633.1 \pm 65.3 \mu\text{m}$), 5 ($682.0 \pm 61.0 \mu\text{m}$), and 10 ($751.3 \pm 38.0 \mu\text{m}$) ng/ml had statistically longer neurite extensions than the control group (0 ng/ml; $469.7 \pm 22.1 \mu\text{m}$; $P < 0.05$) [Figs. (1 and 2)a]. In contrast, there was no difference in neurite length between DRGs exposed to 20 ng/ml FK506, 50 ng/ml FK506, or control. Treatment of the DRGs with NGF concentrations of 0.1 ($910.5 \pm 39.8 \mu\text{m}$), 1 ($993.7 \pm 53.6 \mu\text{m}$), 5 ($1063.5 \pm 57.8 \mu\text{m}$), 10 ($1117.6 \pm 55.6 \mu\text{m}$) ng/ml, and 100 ng/ml ($755.7 \pm 57.8 \mu\text{m}$) enhanced neurite elongation compared with control ($469.7 \pm 22.1 \mu\text{m}$; $P < 0.05$) (Fig. 2b).

Combination Treatment—The combined response of 2 drugs was compared with control and with their individual doses to determine the response. The response was found to be potentiating when the combination treatment resulted in a better response than what was obtained individually. Certain treatment responses were found to be competitive when the combined dosage resulted in reduction of growth compared with the individual response. If

there was no significant change in growth with the combination treatment when compared with single treatments, the response was listed as noncompetitive.

The combined treatment of FK506 and NGF showed increased neurite elongation compared with their corresponding individual doses (Fig. 3b and 3c). The combination of NGF with FK506 treatment was potentiating and showed increasing neurite elongation with increasing NGF concentration except when NGF was combined with 1 ng/ml FK506, which was competitive (Fig. 4a). Higher FK506 concentrations (20–100 ng/ml), while inhibitory on their own, exhibited significantly increased elongation in combination with NGF and GDNF (seen in shaded region of Fig. 4a). The best combinations were found to be 1 ng/ml NGF with 5–10 ng/ml FK506, 100 ng/ml NGF with 50 ng/ml FK506, and 10 ng/ml NGF with 20 ng/ml FK506 ($P < 0.05$).

The combined treatment of FK506 and GDNF showed significant increased neurite elongation (shaded region of Figure 4b) as compared with their individual treatments (Figs. 2a,b). The combined treatment of FK506 and GDNF was potentiating for combinations of 1–10 ng/ml of GDNF with 1–10 ng/ml of FK506 (Fig. 3d,e and 5c), and for combination of GDNF 0.1 ng/ml with FK506 50 ng/ml ($P < 0.05$). All other combinations showed a noncompetitive response ($P > 0.05$).

Combined GDNF and NGF had a potentiating effect and produced significant enhancement of neurite outgrowth as compared with individual treatments, shown in shaded regions of Figure 4c. The plots suggest a strong effect of increasing NGF concentration (0.1, 1, and 10 ng/ml). The average neurite length at the optimal GDNF+NGF concentrations (1 ng/ml NGF combined with 1 or 0.1 ng/ml GDNF) ($P < 0.05$) was 1,000–1,100 μm , which is higher than the 800–950 μm neurite elongation observed with NGF or GDNF alone (Fig. 3i and 5e). Altogether, the results indicate a strong interaction of both growth factors. Statistically, combinations of 1 ng/ml NGF with either 0.1 ng/ml, 1 ng/ml, or 10 ng/ml of GDNF were found to produce the best neurite growth response ($P < 0.05$ when compared with control).

Combination treatment with FK506, NGF, and GDNF produced both potentiating and competitive responses on neurite elongation (Table 1). This triple combination group mediated the greatest neurite elongation when compared with all other treatments with 1 ng/ml of each NGF, GDNF, and FK506 enhancing growth to the greatest extent ($1282.4 \pm 25.1 \mu\text{m}$) ($P < 0.05$). All combinations had a potentiating response except for the combinations with 100 ng/ml of NGF and 100 ng/ml of GDNF ($P > 0.05$), which showed a competitive response.

Neurite Branching

Single Growth Factors—FK506 did not promote neurite branching, because there was no statistical difference in neurite branching between the different concentrations tested ($P > 0.05$) (Fig. 6a). NGF and GDNF induced significant neurite branching in the concentration range of 0.1–100 ng/ml ($P < 0.05$) (Figs. 6b,c). Finally, the neurite branching of the NGF or GDNF treated DRG was generally approximately 50% above that observed with the FK506 treated DRGs.

Combined Growth Factors—As seen in Figure 7a, the average neurite branching due to combined NGF+FK506 treatment was not dose-dependent, and the optimum neurite branching response was observed at the combinations of 0.1 ng/ml and 100 ng/ml of NGF with 10 ng/ml of FK506 (0.3 ± 0.01 – 0.33 ± 0.01) ($P < 0.05$) which was the same as seen with NGF alone (0.34 – 0.35) ($P < 0.05$). There was a noncompetitive interaction for combinations of NGF 0.1–100 ng/ml with 1–50 ng/ml of FK506 (0.28 – 0.33).

Generally, GDNF+FK506 treatment (Fig. 7b) produced a competitive response and reduced neurite branching (0.2 – 0.3) as compared with those obtained with GDNF treatment alone (0.3 – 0.4), except when 5 ng/ml of FK506 was combined with 100 ng/ml of GDNF (0.32 ± 0.01) ($P < 0.05$), which had a noncompetitive response.

Figure 7c shows that the optimum neurite branching (0.34 – 0.35) for combined GDNF+NGF treatment was obtained with 1 ng/ml NGF plus 10 or 100 ng/ml GDNF or with 10 ng/ml NGF plus 10 or 100 ng/ml GDNF ($P < 0.05$). Neurite branching measurements were more NGF dose-dependent than GDNF dose-dependent. Neurite branching displayed a competitive interaction at lower GDNF concentrations and a potentiating interaction at GDNF values >1 ng/ml.

Combined treatment of NGF+GDNF+FK506 did not produce enhancement in neurite branching as compared to individual treatments (Table 2). The combinations of NGF/GDNF (1–10 ng/ml) and FK506 (10 ng/ml) resulted in a noncompetitive response (0.3 – 0.35) ($P < 0.05$) while the combinations involving higher concentrations of NGF and GDNF (100 ng/ml) caused a competitive interaction (0.15 – 0.22) ($P > 0.05$). The response obtained was not dose-dependent.

Neurite Growth Rate—The rate of neurite growth was measured over 3 days (Fig. 8). The concentrations of 10 ng/ml of FK506, NGF, and GDNF were chosen from our analysis of neurite growth at 72 h, because they consistently enhanced neurite growth to a greater extent than controls. The growth rate of the control group slowed down by day 2, such that there was no significant difference in neurite length between days 2 and 3 ($P > 0.05$) (Fig. 8). There was a close to linear increase in neurite length over the 72-h time frame, when DRGs were exposed to FK506, NGF, or a combination of the 2 (Fig. 8a). However, the combination of FK506 and NGF had a steeper growth rate compared with single treatment exposure, as evidenced by the longer neurites at 72 h ($933.2 \pm 21.2 \mu\text{m}$) compared with the other experimental groups (NGF: $789.5 \pm 23.4 \mu\text{m}$, GDNF: $723.1 \pm 22.4 \mu\text{m}$). When DRGs were exposed to GDNF, there was initially a steep increase in neurite growth, but the rate decreased dramatically between days 2 and 3 (Fig. 8b). In contrast, there was a more linear increase in neurite growth when DRGs were exposed to the combination of FK506 and GDNF; this combination had the longest neurites by 72 h ($811.3 \pm 37.4 \mu\text{m}$) (Fig. 8b). For the different groups, the overall daily growth rate over 3 days of incubation was approximately 188.1 ± 21.1 , 228.6 ± 33.2 , 263.2 ± 42.1 , 241.0 ± 37.5 , 316.2 ± 23.4 , 311.1 ± 21.2 , 270.4 ± 25.3 , and $320.4 \pm 30.1 \mu\text{m/d}$ for the control group, the FK506, NGF, GDNF, NGF+GDNF, NGF+FK506, GDNF+FK506, and NGF+GDNF+FK506 treatment groups, respectively.

Western Blot—Based on our neurite growth data, we tested some of the optimum dosages from the single and combined groups for protein expression using Western blot analysis (Fig. 9). The protein expression levels shown represent the ratio of target protein to the GAPDH internal control. Western blots showed Akt expression of 14.2% by DRGs treated with a combination of 1 ng/ml NGF and 10 ng/ml FK506. This interaction was potentiating ($P < 0.05$) when compared with the individual treatments of 1 ng/ml NGF and 10 ng/ml FK506, which resulted in 7.7% and 5% expression of Akt, respectively. Similar results were seen for pAkt expression by the NGF+FK506 treated DRGs (~12%) as compared with 10% pAkt expression by single treatment of 1 ng/ml NGF or ~9% pAkt expression by 10 ng/ml FK506 dose ($P < 0.05$). This result was in agreement with DRG growth readings (Figs. 2a,b, 4a), which showed that combined treatment of NGF+FK506 enhanced neurite growth more than either drug alone.

The optimum GDNF+FK506 (1 ng/ml GDNF+10 ng/ml FK506) combined treatment for neurite elongation had a slightly competitive interaction resulting in AKT expression of ~4%, which was slightly lower than what was observed from a single treatment of 10 ng/ml FK506 (AKT 5%; pAkt 8.5%) or 1 ng/ml GDNF (Akt ~4%; pAkt ~7.7%). This result is similar to what we observed from DRG neurite elongation data for this treatment (Figs. 2a,c, 4b).

The overall response of the optimal combined treatment of NGF+GDNF+FK506 (1 + 1+1 ng/ml) was the highest with Akt expression of ~16% and pAkt expression ~13% when compared with dual combinations or the single treatments ($P < 0.05$). This result was consistent with the neurite growth readings obtained from DRGs (Table 1).

DISCUSSION

The main findings of this study are that: (1) FK506 primarily enhances neurite elongation; (2) NGF and GDNF promote both neurite elongation and neurite branching; (3) neurite outgrowth is dose-dependent, with optimal concentrations falling in the range of 1–10 ng/ml for FK506, NGF, and GDNF; (4) combining FK506, GDNF, and NGF exerts a potentiating effect on neurite growth; (5) significant increases in neurite growth are observed in the first 48 h of culture; and (6) combinations of FK506, GDNF, and NGF increased protein expression of AKT and phosAKT when compared with individual treatments.

NGF and GDNF are important neurotrophins.⁴⁰ They have been shown to promote neuronal growth both *in vitro* and *in vivo*⁹ and have been studied extensively.^{41,42} They are the 2 most commonly used neurotrophins when selecting or designing a delivery system for nerve growth and have been used in numerous neurorestorative studies.^{40–43} NGF has been found to be essential for development and phenotypic maintenance of neurons in the peripheral nervous system.⁴⁴ NGF has been considered to be a very powerful and selective growth factor for sympathetic and sensory neurons, especially for survival and axonal outgrowth of sensory neurons.^{41,45} GDNF has shown pronounced effects on motor axonal regeneration and has been identified as a key factor for promoting axonal elongation.^{40,41,46,47} More recent work has shown that GDNF ligands can regulate pain responses by modulating sensitization of sensory neurons to different stimuli.^{48–50} GDNF was also shown recently to

elicit different effects on motoneuron axons versus sensory cell bodies.⁵¹ Delivery of GDNF in a cell therapy model has been shown to prolong lifespan and control disease progression in a rat model of amyotrophic lateral sclerosis.⁵²

This study analyzed the potential effects of different dosages of FK506 in combination with other growth factors on nerve growth. *In vivo* tests have shown that systemically delivered FK506 has nerve regenerative properties in addition to being an immunosuppressant.^{20,29,30} There is, however, insufficient information regarding the optimal local dose of FK506 for nerve regeneration after injury. To improve this knowledge, we used a bio-activity test system based on DRG explants from chicken embryos. We have tested the effects of FK506, GDNF, and NGF, alone and in combination, on neurite elongation (Figs. 1–4), branching (Figs. 6 and 7), growth kinetics (Fig. 8), and protein expression of AKT and pAKT (Fig. 9). Chicken DRG explants were selected for this testing process because of their availability, low cost, and their ganglion structure associated with Schwann cells. Due to their structure, DRG explants more closely mirror the situation of neurite growth *in vivo* than single neurons.⁴² Chicken DRG explants are a widely used model for neurite growth. Chicken embryonic DRGs are readily available and have also been shown to exhibit growth similarities with other animal neural systems.^{9,37} DRGs have been used for testing effects of neurotrophic factors and drugs in several animal models.⁵³ Neurite outgrowth and survival studies have been done using DRGs.⁵³ We expected that the use of DRGs for testing should provide relevant information on bio-activity and the useful dose of selected drugs. FK506, GDNF, or NGF induced readily visible neurite outgrowth when compared with control cells. FK506 and GDNF primarily promoted neurite elongation, whereas NGF induced extensive neurite branching with neurite elongation. We found that 1–10 ng/ml of FK506, NGF, and GDNF showed the highest level of elongation of DRG neurites.

The dramatic neurotrophic action of combined doses of FK506 and other growth factors on DRGs is extraordinary in terms of sensitivity, because as little as 1 ng/ml FK506 combined with 1 ng/ml NGF/GDNF was neurotrophic. The effect of NGF/GDNF in promoting neurite sprouting and elongation has been shown earlier.⁹ On a molecular level, NGF is known to stimulate neurite extension in embryonic DRG neurons by binding to tyrosine kinase A (TrkA) receptors and activating intracellular signaling pathways,⁵⁴ and GDNF promotes primarily motor neuron survival and neurite growth.⁴⁷ The optimal NGF/GDNF dose of 0.1–10 ng/ml for maximum neurite outgrowth we observed is in agreement with previous data.⁹

Although neurite growth did not differ significantly between treatments with 1 and 10 ng/ml of GDNF or NGF, it declined at higher doses (100 ng/ml). This result is in agreement with findings in rats, where high doses of NGF delayed nerve regeneration by retarding GAP43 production in the early phase (7 days) after axotomy.¹⁵ We observed that combinations of FK506 with high doses of NGF produced a potentiating response in neurite elongation, which suggests that FK506 may help to enhance neurite growth even with higher doses of neurotrophic factors. In certain cases of combined doses, such as combinations of 1 ng/ml or 5 ng/ml FK506 with GDNF, we see a competitive interaction which could reflect negative feedback resulting from the interaction of different protein expressions and signaling pathways used by FK506 and the neurotrophic factors.⁵⁵

No difference in the rate of neurite outgrowth was found between FK506, GDNF, and NGF treatment during the first 2 days of treatment, but NGF yielded a higher outgrowth rate than others between days 2 and 3 of the experiment. Interestingly, the combination of FK506 with GDNF and/or NGF enhanced the rate of neurite outgrowth at all time points over the other treatment groups. The potentiating effect of FK506 with GDNF and/or NGF on initial neurite growth rate should be considered when developing new therapeutic drug delivery systems.

The potentiating effect of FK506 with GDNF and/or NGF on both neurite growth and, to a lesser extent, on neurite branching is an interesting observation. NGF and GDNF have been found to activate Akt and pAkt expression in cells in neuroprotection and neuro-regeneration pathways.^{41,44} Akt is a serine-threonine protein kinase. Induction of the serine-threonine kinase activity is critical for cell survival, as well as cell proliferation. Akt is also regulated by growth factor and serum factor signaling through PI3 kinase.^{32,36} Data suggest that FK506 potentiates neurite outgrowth through the Ras/Raf/MAP kinase signaling pathway downstream of PI3K-Akt.²⁸ The phosphatidylinositol-3 kinase–Akt (PI3K-Akt) pathway is one of the major growth factor pathways that has been studied and shows that Akt/pAkt expression is associated with accelerated neurite regeneration after axotomy.^{56,57}

Also, Akt has been found to be necessary for neurotrophin-stimulated neurite growth.^{36,56} With Western blot analysis, we found that Akt/pAkt activation occurs during neurite growth, and the percentage of protein expression can be related to neurite elongation due to different dosage combinations. In this study, we evaluated whether FK506 individually or in combination with other neurotrophic factors would upregulate the protein expression of Akt and phosAkt. We found that combined application of neurotrophic factors with FK506 enhanced co-expression of both receptors. These results strongly suggest that the interaction of FK506 with GDNF and NGF mediates distinct neurite growth enhancement. Activation of the Akt signaling pathway has been associated with increased neurite growth⁵⁸ and would explain the increased expression of receptors.

In conclusion, these results indicate that there is a dramatic increase in nerve growth activity when FK506 is combined with NGF and/or GDNF. NGF and FK506 combined treatments showed potentiating responses for certain dose combinations and had significant effects on neurite growth when compared with their individual doses. Combined GDNF and FK506 treatments exhibited a slightly potentiating effect, but to a lesser extent when compared with FK506+NGF. Combined treatments of all 3 drugs were highly potentiating and resulted in extraordinary effects on neurite growth and branching. However, further investigations are required to elucidate the mechanisms that support the enhanced neurite elongation by combined FK506 and GDNF/NGF treatment. These neurotrophic actions have positive therapeutic ramifications. Numerous neurotrophic proteins, such as brain derived neurotrophic factor, GDNF, and NGF, are being evaluated clinically in various disease states. Difficulties in manufacturing of these large proteins and delivery to target sites create serious barriers to therapeutic application. Drugs such as FK506 are readily synthesized and are thermally stable. Both NGF and GDNF are synthesized endogenously in low amounts at the site of nerve injury, and their efficacy may be enhanced with exogenous delivery of FK506.

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Abbreviations

| | |
|--------------|---|
| BSA | bovine serum albumin |
| DMEM | Dulbecco's modified eagle medium |
| DRG | dorsal root ganglion |
| FBS | fetal bovine serum |
| FK506 | tacrolimus |
| GDNF | glial cell line derived neurotrophic factor |
| NGF | nerve growth factor |
| PBS | phosphate buffered saline |

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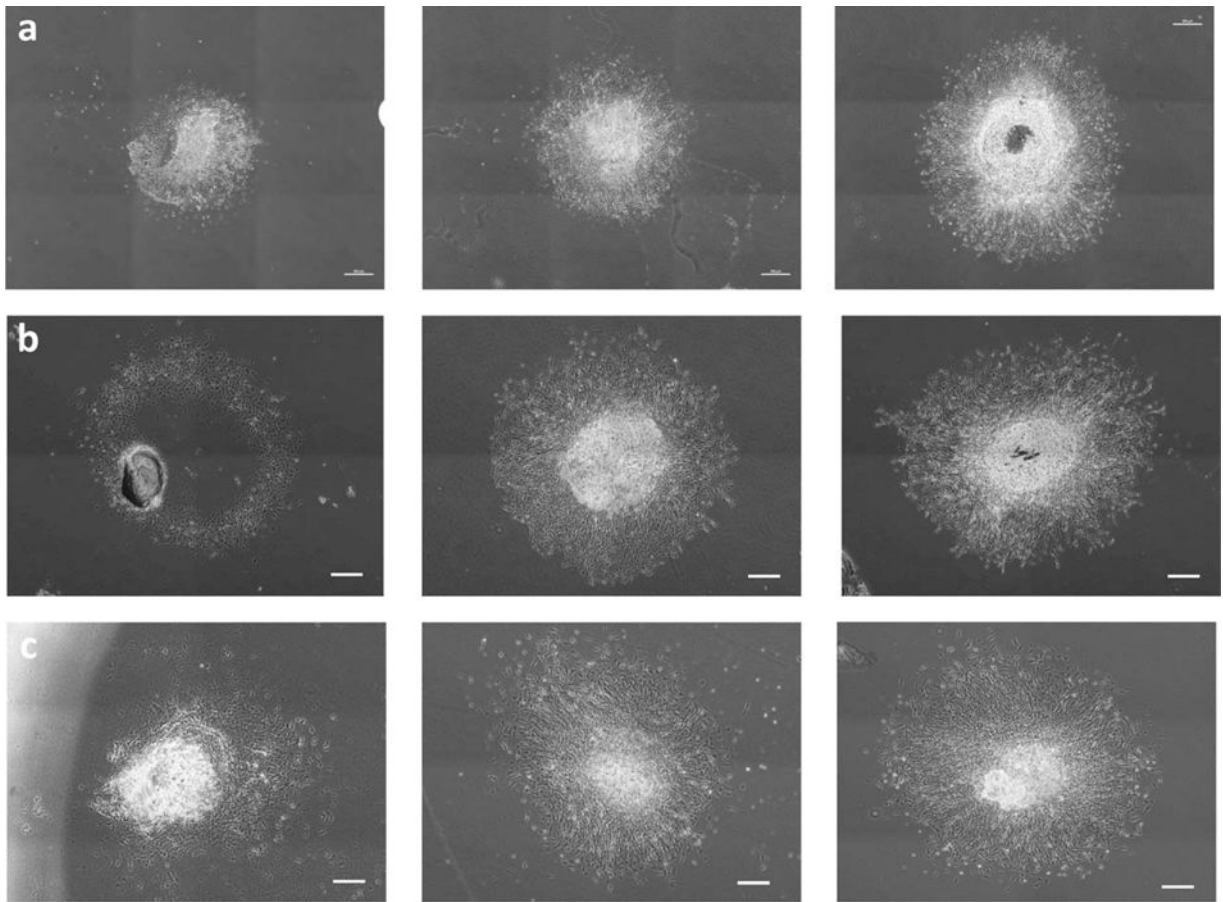


FIGURE 1.

Phase contrast images of DRGs treated with different concentrations of FK506: control (left), 1 ng/ml (center), 10 ng/ml (right) (a); NGF: control (left), 1 ng/ml (center), 10 ng/ml (right) (b); GDNF: control (left), 1 ng/ml (center), 10 ng/ml (right) (c). Neurite outgrowth can be seen based on different doses of FK506, NGF, and GDNF. Scale = 500 μ m.

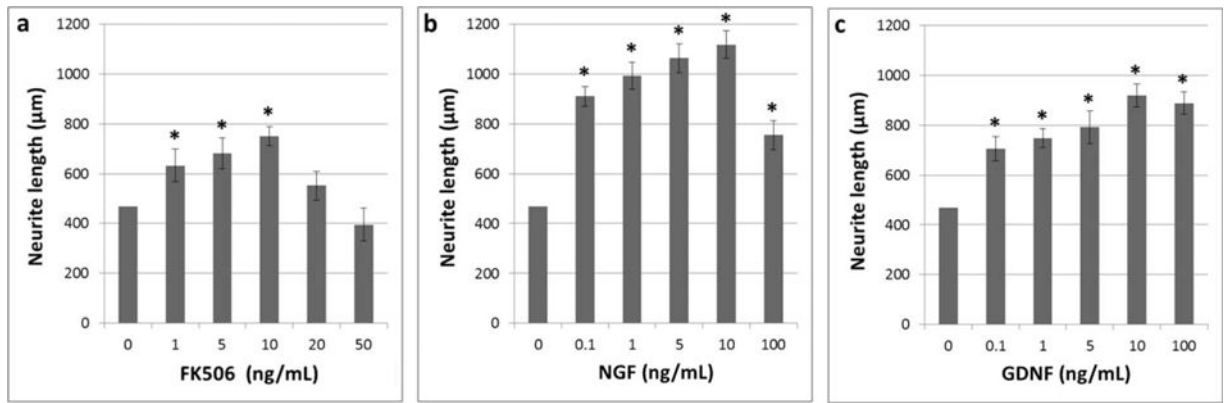


FIGURE 2.

Plots showing effect of single drug treatments on axonal elongation. (a) FK506 Dosage/length. (b) NGF Dosage/length. (c) GDNF Dosage/length. The bars represent mean \pm SD for $n = 4$ (* statistically different from 0 ng/ml; $P < 0.05$).

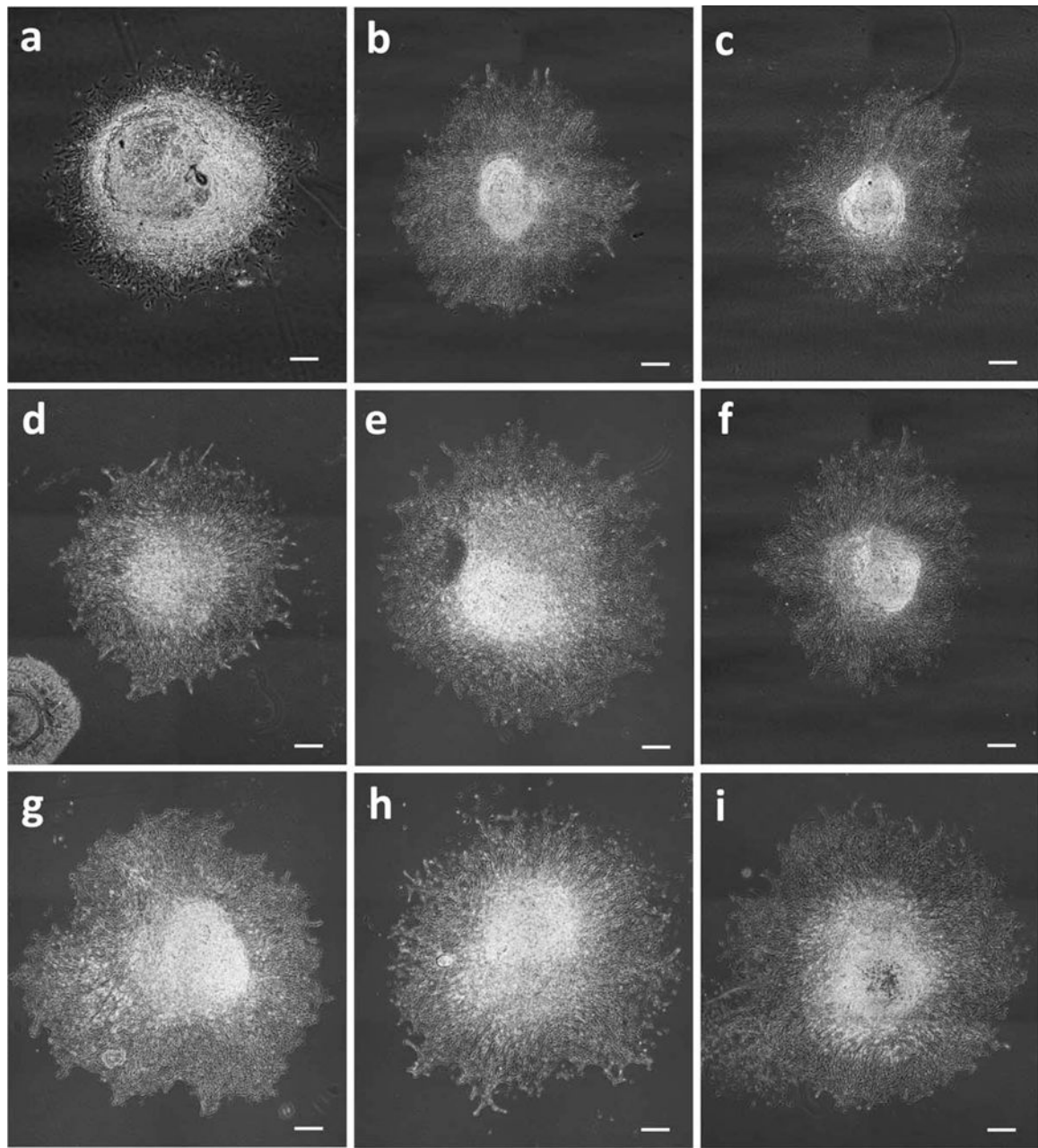


FIGURE 3.

Phase contrast images of DRGs treated with growth factor combinations (starting from top left). **(a)** control (0 ng/ml). **(b)** NGF 1 ng/ml + FK506 1 ng/ml. **(c)** NGF 1 ng/ml + FK506 10 ng/ml. **(d)** GDNF 1 ng/ml + FK506 1 ng/ml. **(e)** GDNF 1 ng/ml + FK506 10 ng/ml. **(f)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 1 ng/ml. **(g)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 10 ng/ml. **(h)** NGF 10 ng/ml + GDNF 10 ng/ml + FK506 10 ng/ml. **(i)** NGF 1 ng/ml + GDNF 1 ng/ml. Neurite outgrowth is increased with combined treatment of FK506 with growth factors. Scale = 500 μ m.

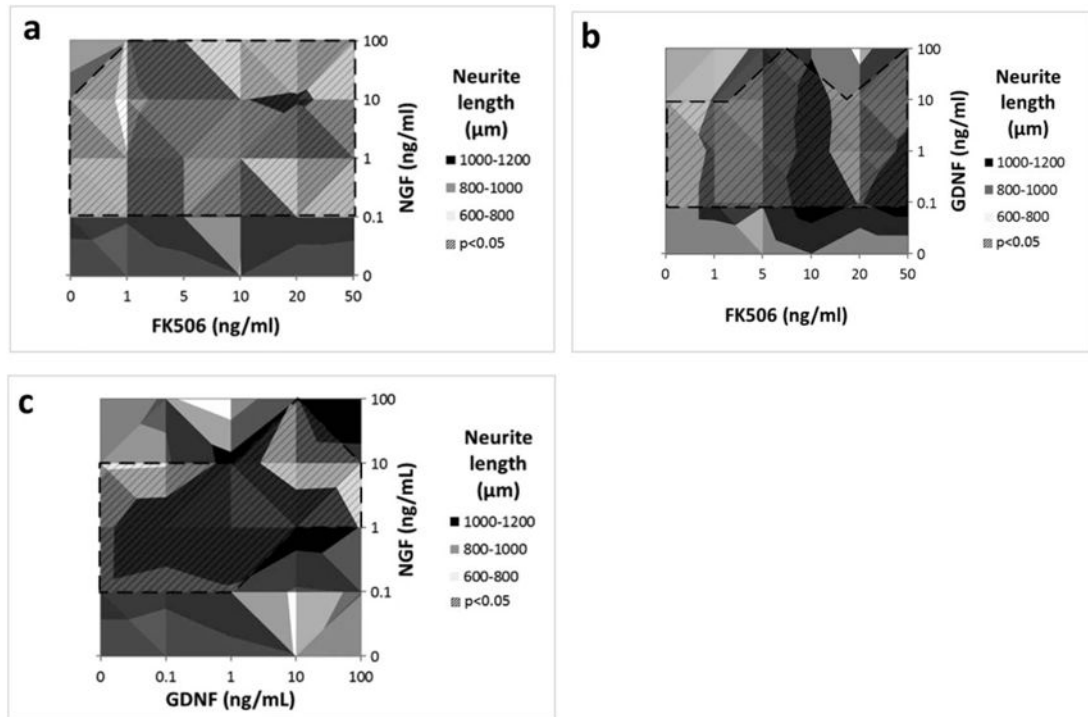
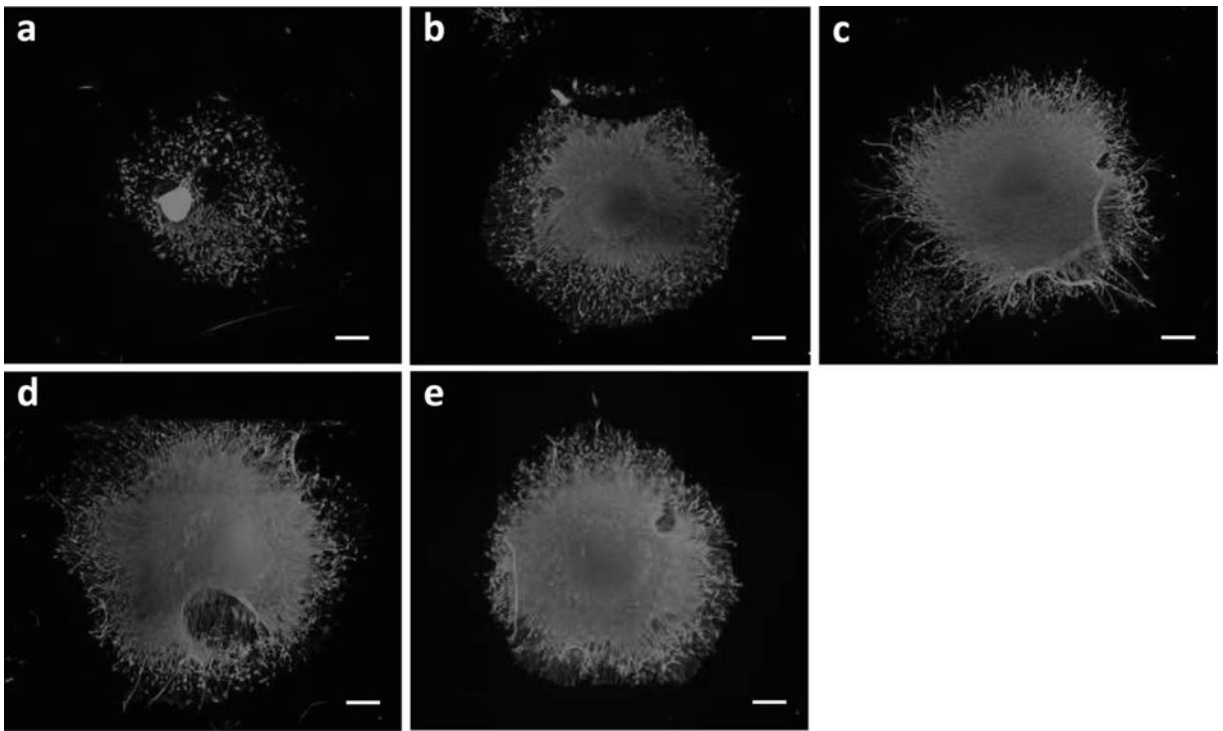


FIGURE 4.

Surface plots showing effect of combined drug treatments on axonal elongation. (a) NGF +FK506 combined dosage. (b) GDNF+FK506 combined dosage. (c) NGF+GDNF combined dosage. (Shaded region with dashed lines specifies the concentration range which produced significantly different axonal elongation response from 0 ng/ml, $P < 0.05$).

**FIGURE 5.**

Fluorescence images of DRGs stained with β -tubulin. **(a)** control (0 ng/ml). **(b)** NGF 1 ng/ml + FK506 10 ng/ml. **(c)** GDNF 1 ng/ml + FK506 10 ng/ml. **(d)** NGF 1 ng/ml + GDNF 1 ng/ml + FK506 1 ng/ml. **(e)** NGF 1 ng/ml + GDNF 1 ng/ml. The images show enhanced neurite elongation with the specified treatments.

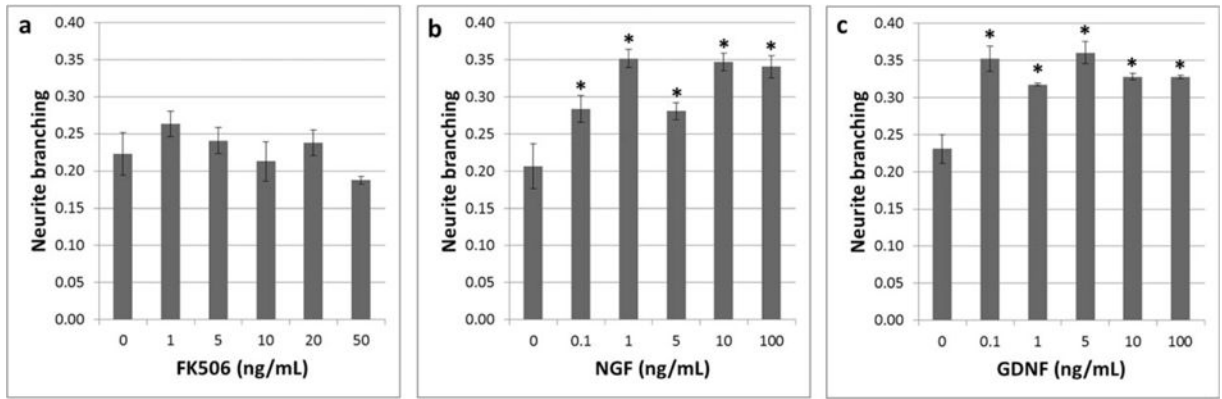


FIGURE 6.

Plots showing the effect of single drug treatment on axon density. **(a)** FK506 Dosage/density. **(b)** NGF dosage/density. **(c)** GDNF dosage/density. The bars represent mean \pm SD for $n = 4$ (*statistically different from 0 ng/ml; $P < 0.05$).

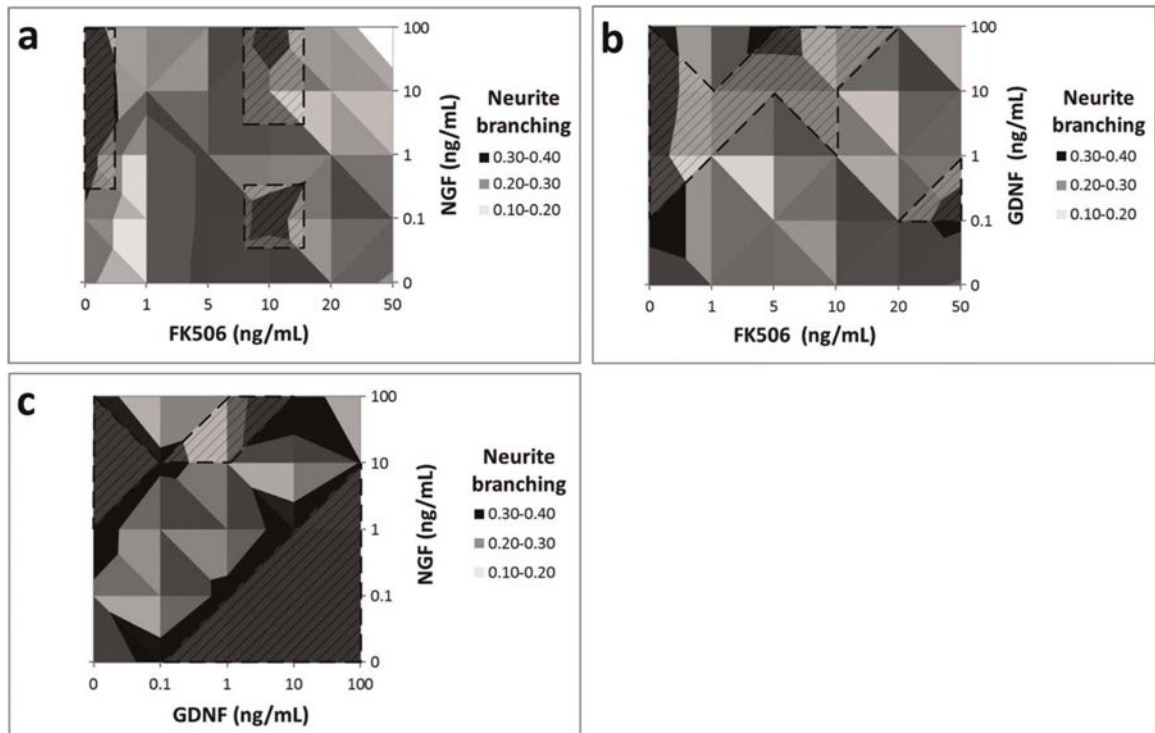
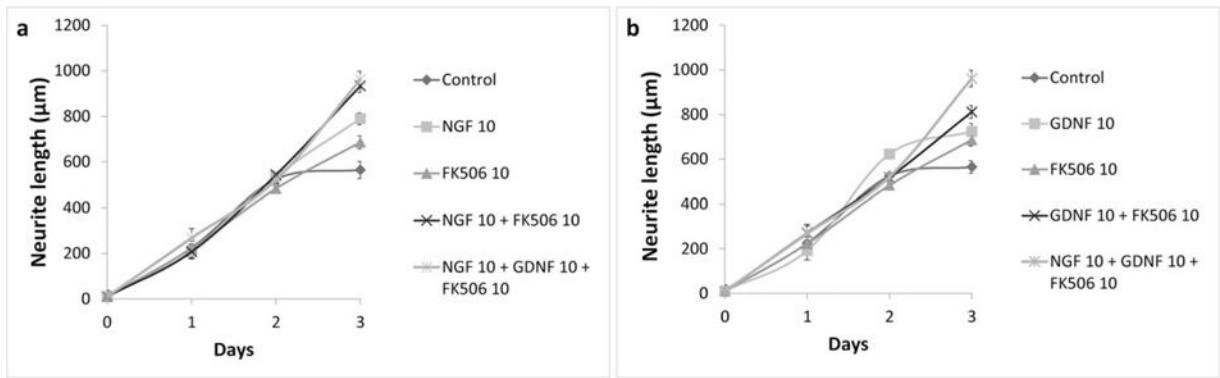


FIGURE 7. Surface Plots showing the effect of combined drug treatment on axon density. **(a)** NGF +FK506 combined dosage. **(b)** GDNF+FK506 combined dosage. **(c)** NGF+GDNF combined dosage. Shaded region with dashed lines specifies the concentration range that produced significantly different axonal branching responses when compared with 0 ng/ml, $P < 0.05$.

**FIGURE 8.**

Kinetics of axonal elongation of DRGs. **(a)** treated with FK506 alone (10 ng/ml), NGF alone (10 ng/ml), combined NGF/FK506 (10 ng/ml each), combined NGF/GDNF/FK506 (10 ng/ml each), and untreated (control). **(b)** treated with GDNF alone (10 ng/ml), FK506 alone (10 ng/ml), combined GDNF/FK506 (10 ng/ml each), combined NGF/GDNF/FK506 (10 ng/ml each), and untreated (control). Bars = mean \pm SD of $n = 3$. All treatment groups were significantly different ($P < 0.05$) from control (not indicated with asterisks).

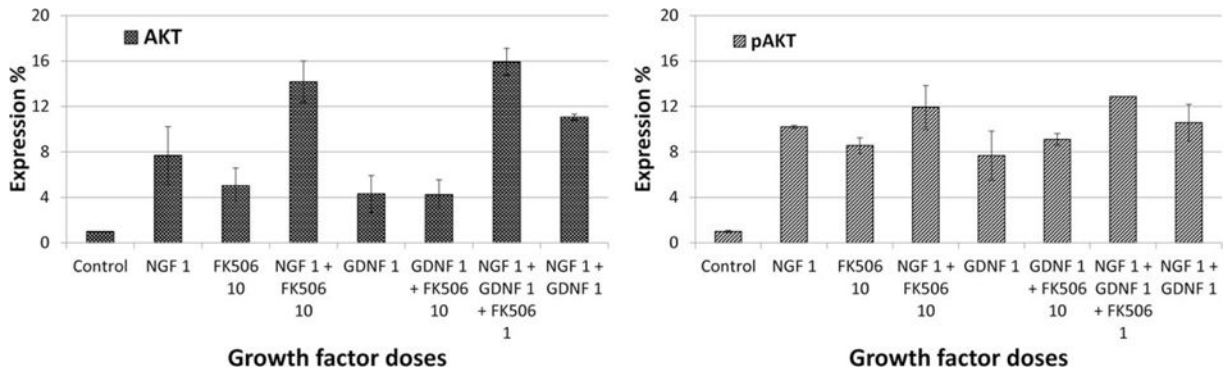


FIGURE 9. Plots showing protein expression of DRG lysates treated with different drug treatments. The bars indicate mean \pm SD for $n = 3$ obtained from densitometry of western blot scans.

Table 1

Effect of NGF+GDNF+FK506 combined dosage on neurite elongation. *

| FK506 (ng/ml) | NGF (ng/ml) | GDNF (ng/ml) | Neurite length (μm) |
|---------------|-------------|--------------|----------------------------------|
| 0 | 0 | 0 | 417.4 |
| 1 | 1 | 1 | 1282.4 |
| 1 | 5 | 10 | 834.1 |
| 1 | 10 | 5 | 765.3 |
| 5 | 5 | 100 | 966.6 |
| 5 | 10 | 1 | 889.1 |
| 5 | 100 | 5 | 781.6 |
| 10 | 1 | 1 | 905.7 |
| 10 | 5 | 1 | 843.8 |
| 10 | 10 | 10 | 1025.7 |
| 10 | 10 | 10 | 1036.1 |
| 20 | 1 | 10 | 1005.7 |
| 20 | 100 | 100 | 743.8 |

* All of the combinations listed were significantly different from 0 ng/ml, $P < 0.05$.

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Table 2

Effect of NGF+GDNF+FK506 combined dosage on neurite branching

| FK506 (ng/ml) | NGF (ng/ml) | GDNF (ng/ml) | Neurite branching |
|---------------|-------------|--------------|-------------------|
| 0 | 0 | 0 | 0.21 |
| 1 | 1 | 1 | 0.35 |
| 1 | 5 | 10 | 0.36 |
| 1 | 10 | 5 | 0.30 |
| 5 | 5 | 100 | 0.33 |
| 5 | 10 | 1 | 0.33 |
| 5 | 100 | 100 | 0.25 |
| 5 | 100 | 5 | 0.31 |
| 10 | 1 | 1 | 0.16 |
| 10 | 5 | 1 | 0.34 |
| 10 | 10 | 10 | 0.33 |
| 10 | 10 | 10 | 0.30 |
| 20 | 1 | 10 | 0.35 |

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