FK962 Promotes Neurite Elongation and Regeneration of Cultured Rat Trigeminal Ganglion Cells: Possible Involvement of GDNF

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Purpose. Amputation of the trigeminal nerve leads to decreased corneal sensitivity and dry eye. Our previous study showed that the drug FK962 (N-[1-acetylpiperidin-4-yl]-4-fluorobenzamide) induced neurite elongation from trigeminal ganglion (TG) cells and accelerated recovery of corneal sensitivity in a rabbit model of in situ keratomileusis (LASIK) surgery. However, the molecular pathways leading to FK962-induced neurite elongation and regeneration are not well defined. Thus, the purposes of the present experiments were to determine if FK962 induces elongation and regeneration of cultured rat TG cells and to investigate the mechanism of FK962-induced neurite elongation.

Methods. Mixed TG cells were cultured with or without FK962, and immunocytochemistry was used to detect stimulation of neurite elongation. Neurite regeneration was also tested in an in vitro model of neuronal ablation. ELISA was used to detect glial cell line–derived neurotrophic factor (GDNF) and somatostatin (SST) release, and mRNA expression was measured by qPCR. Antibody neutralization was used to determine the mechanism for FK962-induced neurite elongation/regeneration.

Results. FK962 enhanced elongation and regeneration of neurites in TG neurons. GDNF treatment–induced neurite elongation and GDNF antibody significantly inhibited neurite elongation induced by GDNF and FK962. Nerve growth factor (NGF) treatment also induced neurite elongation, which was inhibited by NGF antibody, but NGF antibody did not inhibit FK962-induced neurite elongation.

Conclusions. Our data suggested that FK962 stimulated induction of GDNF from TG cells. GDNF may be a part of the signaling pathway for FK962-induced neurite elongation/regeneration in rat TG neurons. (*Invest Ophthalmol Vis Sci.* 2012;53:5312–5319) DOI:10.1167/iovs.11-8957

Denervation of the cornea can lead to dry eye, which may occur after in situ keratomileusis (LASIK) used to correct myopia.[1,2] Sensory nerves in the subbasal and the superficial stromal regions of the cornea are amputated by the microkeratome in creating the cornel flap. This leads to nerve degeneration and a decrease in corneal sensitivity.[1] For example, the density of subbasal nerves in the cornea was significantly decreased 1 month after LASIK surgery, then gradually recovered; but some patients had not returned to pre-LASIK levels even after 3 years.[3,4] Such patients can exhibit reduced tear secretion, since the corneal nerves are involved in feedback loop stimulation of tear secretion via autonomic nerves innervating the lacrimal gland (Fig. 1A).[5]

In the corneal nerves, Aδ fibers are the major corneal neurons activated by mechanical stimuli,[6] and they are therefore especially relevant to the current interest in corneal sensitivity and dry eye. Indeed, our recent data showed that recovery of corneal sensitivity measured by mechanical excitation was well correlated with the regeneration of Aδ fiber after flap surgery.[7]

Previous studies showed that nerve growth factor (NGF) caused neuronal outgrowth in trigeminal neurons,[8] and NGF had beneficial effects on the early recovery of corneal sensitivity after LASIK.[9] Pituitary adenylate cyclase-activating polypeptide (PACAP) also induced outgrowth of neuronal processes in trigeminal ganglion (TG) cells, and PACAP accelerated recovery of corneal sensitivity after creation of a corneal flap in rabbits.[10] Thus, drugs that stimulate bioactive neuropeptides—such as glial cell line–derived neurotrophic factor (GDNF)—involved in corneal regeneration would be potentially useful in therapy for dry eye caused by LASIK surgery.

One such drug is FK962 (N-[1-acetylpiperidin-4-yl]-4-fluorobenzamide; Fig. 1A). Recently, we reported that FK962 induced neurite elongation in cultured rabbit TG cells, and FK962 accelerated regeneration of amputated axons and recovery of corneal sensitivity in an in vivo model of LASIK surgery in rabbits.[7] An analog of FK962 caused production of GDNF in cultured rat astrocytes.[11] GDNF promotes survival and axonal regeneration in a wide variety of neuronal populations.[12,13] FK962 also enhanced release of somatostatin (SST) from rat hippocampal slices,[14] and SST promoted neurite outgrowth in rat cerebellar granule cells.[15] The molecular mechanism for the action of FK962 on neurite elongation and axonal regeneration is not yet well defined. Therefore, the specific purposes of the present studies were to determine if FK962 induces elongation of cultured rat TG cells, test if FK962 stimulates elongation of amputated TG cells in an in vitro model of neuronal ablation, and investigate the mechanism of FK962-induced neurite elongation.

Materials and Methods

Animals

Sprague-Dawley rats at 2 weeks of age were obtained from Japan SLC (Shizuoka, Japan). All experimental animals were handled in accor-

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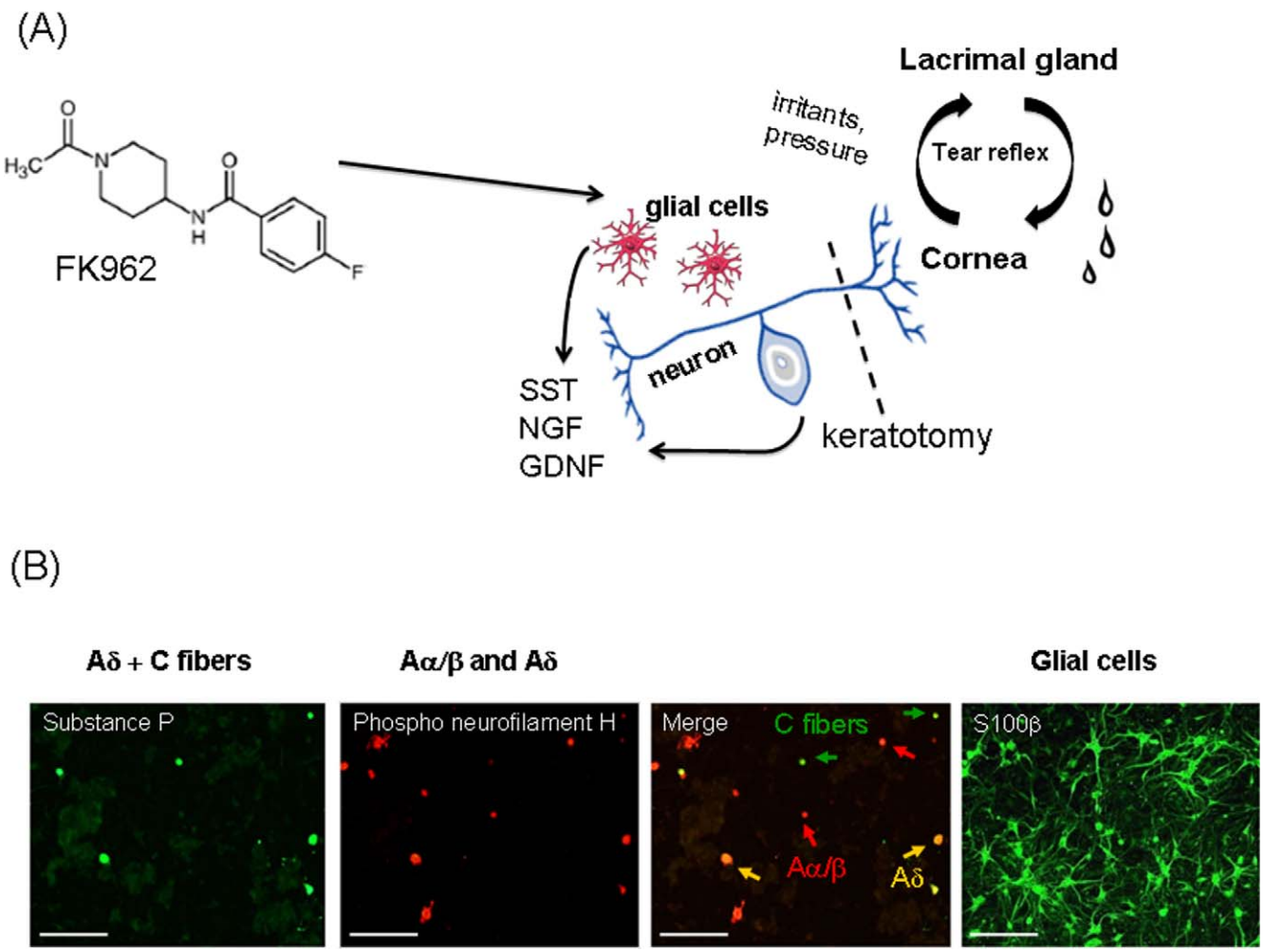

Figure 1. (**A**) Structure of FK962 and a diagram postulating how FK962 may influence the tear reflex through the sensory fibers in the TG nerve complex. (**B**) Immunocytochemistry of our cultured mixed TG cells system showing: (*first panel*), substance P staining (*green*) for Aδ and C fiber neurons; (*second panel*) phospho neurofilament H staining (*red*) for Aα/β and Aδ neurons; (*third panel*) merging of the first two panels to locate individual Aδ neurons (*orange arrows*), Aα/β neurons (*red arrows*), and C fiber neurons (*green arrows*); and (*fourth panel*) S100β-positive (*green*) for glial cells abundant in the TG cultures. Scale bar = 200 μm.

dance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). This study was approved by the Institutional Animal Care and Use Committee of Senju Pharmaceutical Co., Ltd.

Isolation and Culture of TG Cells from Trigeminal Ganglion

After rats were euthanized, the brain and trigeminal ganglia were extracted and rinsed in cold Hanks' balanced salt solution (HBSS; Invitrogen, Life Technologies, Carlsbad, CA). TG cells were prepared according to a previously reported method with modifications.[10] Briefly, the trigeminal ganglia were minced with scissors and digested for 30 minutes with 3 mg/mL collagenase A (Roche Diagnostics, Basel, Switzerland), and then incubated for 40 minutes in neuronal dispersion liquid (Sumitomo Bakelite, Tokyo, Japan) at 37°C. Following filtration through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ), 10% BSA solution was added, and the cells were pelleted by centrifugation (30 minutes, 1000*g*). Mixed TG cells containing neurons and glial cells were seeded at 2.5×10^5 neuronal cells/well into 8-well dishes or at 2.0×10^3 neuronal cells/well into 35-mm glass-bottom dishes, coated with poly-D-lysine and laminin (Becton Dickinson). The culture medium was neurobasal medium (NB, Invitrogen) supplemented with B-27 (Invitrogen); 1 mM L-glutamine (Invitrogen); primocin (InvivoGen, San Diego, CA); and 10 μM cytosine β-D-arabinofuranoside (Ara-C; Wako, Osaka, Japan). The cells were cultured for 24 hours with or without FK962 (kindly provided by Astellas Pharma Inc., Tokyo, Japan) at 37°C under 5% CO_2 and 95% air. The cells were also treated with GDNF, SST (Merck, Darmstadt, Germany) or NGF (R&D Systems, Minneapolis, MN). Goat GDNF antibody (R&D Systems) at 1 μg/mL or goat NGF antibody (Abcam, Cambridge, UK) at 0.3 μg/mL were used to inhibit GDNF-, NGF-, and FK962-induced elongation, with normal goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/mL or at 0.3 μg/mL used as a negative controls. For qPCR, mixed TG cells were seeded at 2.8×10^4 neuronal cells/well into 6-well dishes and cultured for 17 hours under the condition described above.

Immunocytochemistry

Immunocytochemistry was performed using an antibody for phospho-neurofilament H (Millipore, Bedford, MA) to detect Aα/β/δ neurons, and an antibody for substance P (ImmunoStar, Hudson, WI) to detect Aδ and C fiber neurons. An antibody for S100β (Abcam) was the marker used for glial cells, including satellite and Schwann cells, that are predominant in trigeminal ganglion. Cultured cells were fixed for 20

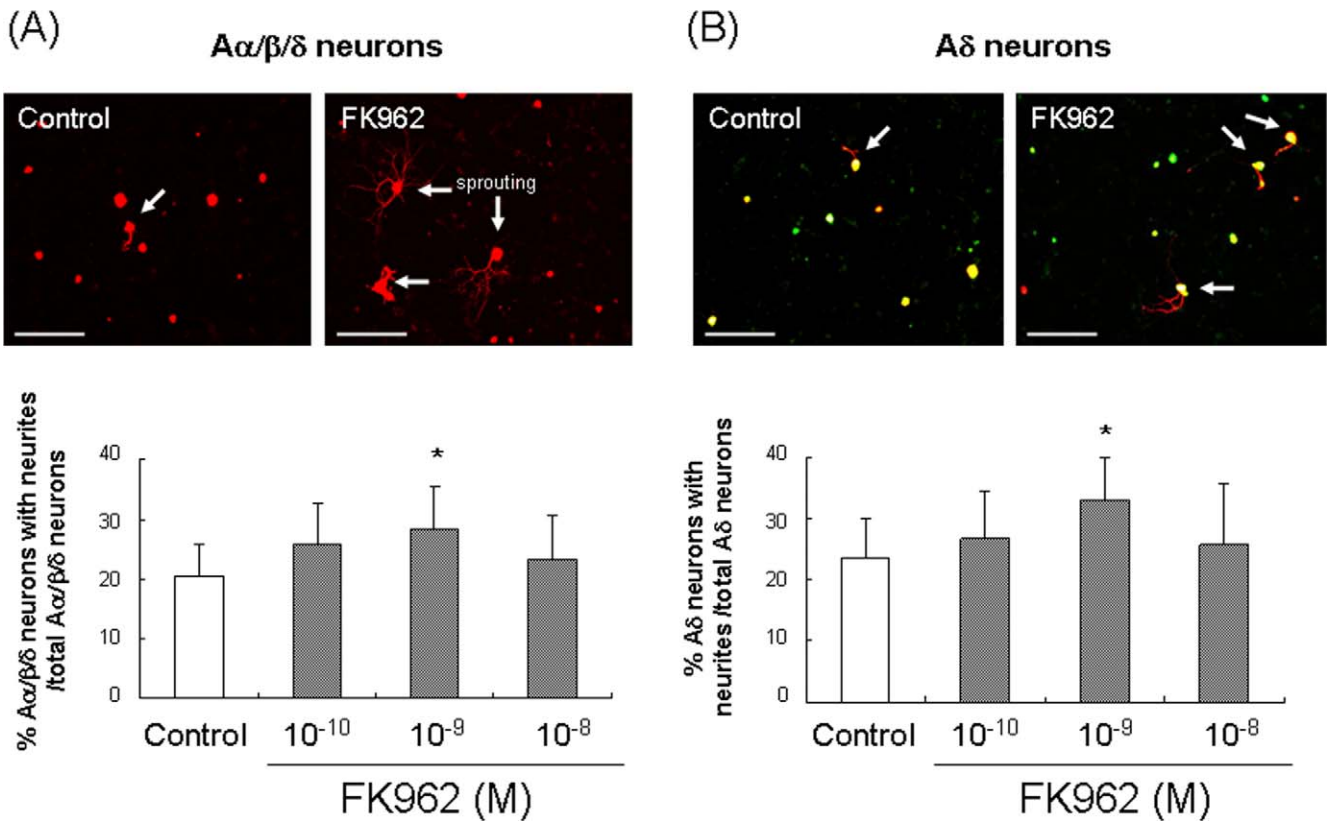

Figure 2. Representative photomicrographs (*upper*) and quantitative analysis (*lower*) showing increase in neurite elongation (*white arrows*) by 10^{-9} M FK962 in: (**A**) Aα/β/δ neurons. (**B**) Aδ neurons. Data are means ± SD ($n = 11$). *$P < 0.05$ relative to control using Dunnett's test.

minutes in 2% formaldehyde solution and permeabilized for 30 minutes with 0.1% Triton X-100 in 5% normal goat serum (Invitrogen). The cells were then incubated for 1 hour with primary antibodies for substance P (1:1000); phospho-neurofilament H (1:1000); or S100β (1:100). After rinsing with PBS, the cells were incubated for 1 hour with goat anti-mouse Alexa Fluor 568 (1:1000; Invitrogen) or goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen). All steps were carried out at room temperature. Stained cells were viewed with a fluorescence microscope (IX71; Olympus, Tokyo, Japan) and digitalized with a charge-coupled device camera.

Assay for Neurite Elongation

Quantitative assessment of neurite elongation was performed by cell counting with image analysis software (Image-Pro Plus; Nippon Roper, Tokyo, Japan). Aα/β/δ neurites exhibiting outgrowth were defined as those with a cell body staining positive for phospho-neurofilament H and an outgrowth process whose length was greater than or equal to the diameter of the cell body. Aδ neurons were specifically identified by positive staining with antibodies for both substance P and phospho-neurofilament H. Data were expressed as percentage of cells with an elongated neurite compared with the total phospho-neurofilament H-positive neuronal cells or double positive Aδ neurons. This neurite elongation assay was appropriate for testing the effect of FK962, because the total number of phospho-neurofilament H-positive neuronal cells, or double positive Aδ neurons were not changed by treatment with FK962.

Assay for Neurite Regeneration

To mimic regeneration of corneal nerves in vivo, mixed TG cells were cultured for 48 hours with 10^{-8} M NGF in NB medium to cause elongation and formation of neuronal networks. A cut approximately 270-μm wide was then made across the network by scratching with tweezers. The cells were washed with PBS to remove detached cells and then cultured for an additional 24 hours with or without 10^{-9} M FK962 in NB medium. After staining for Aα/β/δ neurons, neurite lengths were measured, and neurite regeneration was quantified utilizing Neurocyte software (Kurabo, Osaka, Japan).

Isolation of Neurons and Glial Cells

Neurons were isolated from mixed TG cells according to a modified method reported by Goldenberg et al.[16] TG cells suspended in NB medium were stacked onto a gradient composed of 25 and 50% Percoll (Sigma, St. Louis, MO), and then centrifuged for 10 minutes at 700*g*. Neuronal cells segregating between the two Percoll layers were collected and seeded in NB medium at $5.8–10.0 \times 10^4$ cells onto poly-D-lysine/laminin-coated, 6-well dishes, and cultured for approximately 17 hours.

Glial cells and fibroblasts segregating in the layer between 0% and 25% Percoll were collected, and the glial cells were further isolated according to a modified method reported by Hirata et al.[17] Collected cells were cultured on fibronectin-coated dishes in DMEM/F12 containing 10% FBS (Invitrogen); 2 μM forskolin (Sigma); 10 ng/mL heregulin β1 (Pepro Tech, Rocky Hill, NJ); and 1% penicillin-streptomycin (Invitrogen). After 6 hours, fibroblasts adhered well to the dishes, while glial cells adhered poorly and were readily collected by pipetting. The glial cells were then cultured until confluent on poly-D-lysine/laminin-coated dishes using the same medium as described above. Cells were then subcultured at 10^6 cells/well for 17 hours on poly-D-lysine/laminin-coated 6-well dishes.

RNA Extraction and qPCR

Isolated brain was dissociated manually with a glass homogenizer in TRIzol reagent (Invitrogen), and total RNA was extracted according to

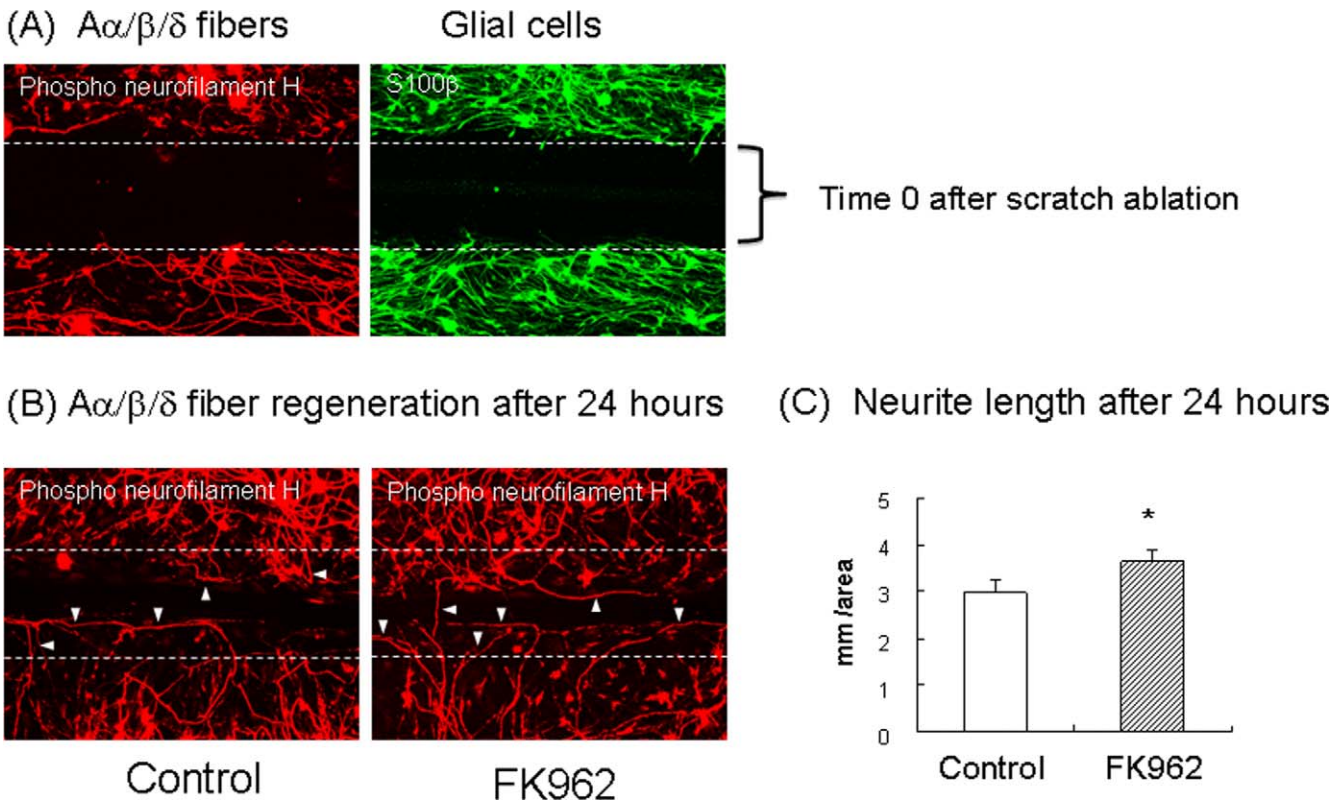

Figure 3. (**A**) Immunofluorescence images for Aα/β/δ neurons (*red*) and glial cells (*green*), at time 0. (**B**) Induction of neurite regeneration after 24 hours. (**C**) Quantitative analysis showing increased neurite lengthening caused by 10^{-9} M FK962. Arrowheads indicate regenerated neurites growing into scratched areas. Scale bar = 200 μm. Data are means ± SD ($n = 5$). $^{*}P < 0.05$ relative to control using Student's *t*-test.

the manufacturer's protocol. Cultured mix TG cells, neuronal, and glial cells were also extracted with TRIzol reagent. One μg of DNase-treated RNA (DNA-*free*, Applied Biosystems, Life Technologies, Carlsbad, CA) was reverse transcribed in 1 × first-strand buffer (Invitrogen) containing 10 mM DTT, 500 μM each dNTP, 10 U/μL SuperScript II, and 2.5 μM random decamers (Applied Biosystems) according to the manufacturer's instructions. First strand cDNA was synthesized at 42°C for 50 minutes and 70°C for 15 minutes.

Gene transcripts for rat GDNF (Assay ID# Rn00569510_m1) and GDNF family receptor alpha-1 (GFRα1, Assay ID# Rn01444617_m1) were quantified by real-time qPCR using TaqMan gene expression assays (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Assay ID# Rn99999916_s1, Applied Biosystems) was used as a reference gene. The PCR reaction was run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute after an initial activation of 50°C for 2 minutes and 95°C for 10 minutes, with 50 ng reverse-transcribed RNA in TaqMan gene expression master mix (Invitrogen). Each qPCR reaction was run in triplicate on a real-time PCR system (Applied Biosystems 7300 or 7500 Real-Time PCR System; Applied Biosystems). Amounts of gene transcripts in samples were determined by comparing unknown cDNA samples with a standard curve made from serial dilutions of known purified PCR products. Data were expressed as relative amounts compared with control samples after normalization with the reference gene, GAPDH.

ELISA for Measurement of GDNF and SST

GDNF (Promega, Madison, WI) and SST (Phoenix Pharmaceuticals, Burlingame, CA) released into the culture medium from mixed TG cells were measured using ELISA kits following the manufacturer's protocol.

Statistical Analysis

Statistical differences ($P < 0.05$) were determined by Dunnett's multiple comparison test (JMP statistical software, SAS Institute, Japan) or Student's *t*-test.

Results

FK962-Induced Neurite Elongation

In our primary cultures of mixed rat TG cells, we observed substance P–positive cells (green, Fig. 1B) indicating Aδ or C nociceptive fiber neurons, phospho-neurofilament H–positive cells (red) indicating Aα/β neurons and Aδ neurons, and double-labeled Aδ neurons (orange arrows).[18,19] As expected, glial cells staining green by antibody for S100β were very abundant (Fig. 1B, far-right panel).

After 24 hours of culture, elongated neurites projected from the somata of Aα/β/δ neurons (Fig. 2A, white arrows) and Aδ neurons (Fig. 2B, white arrows) in controls and more abundantly in the FK962-treated groups. Treatment with FK962 produced a bell-shaped dose response curve, with 10^{-9} M significantly increasing elongation of neurites in Aα/β/δ neurons or Aδ neurons (Figs. 2A, 2B, bottom). The effect of FK962 at higher concentrations was not due to toxicity, because cell numbers were not changed during the experiment (data not shown).

FK962-Induced Neurite Regeneration

Cultured TG cells with NGF-induced neurite networks and scratch ablation showed no neurons (Fig. 3A, left) or glial cells

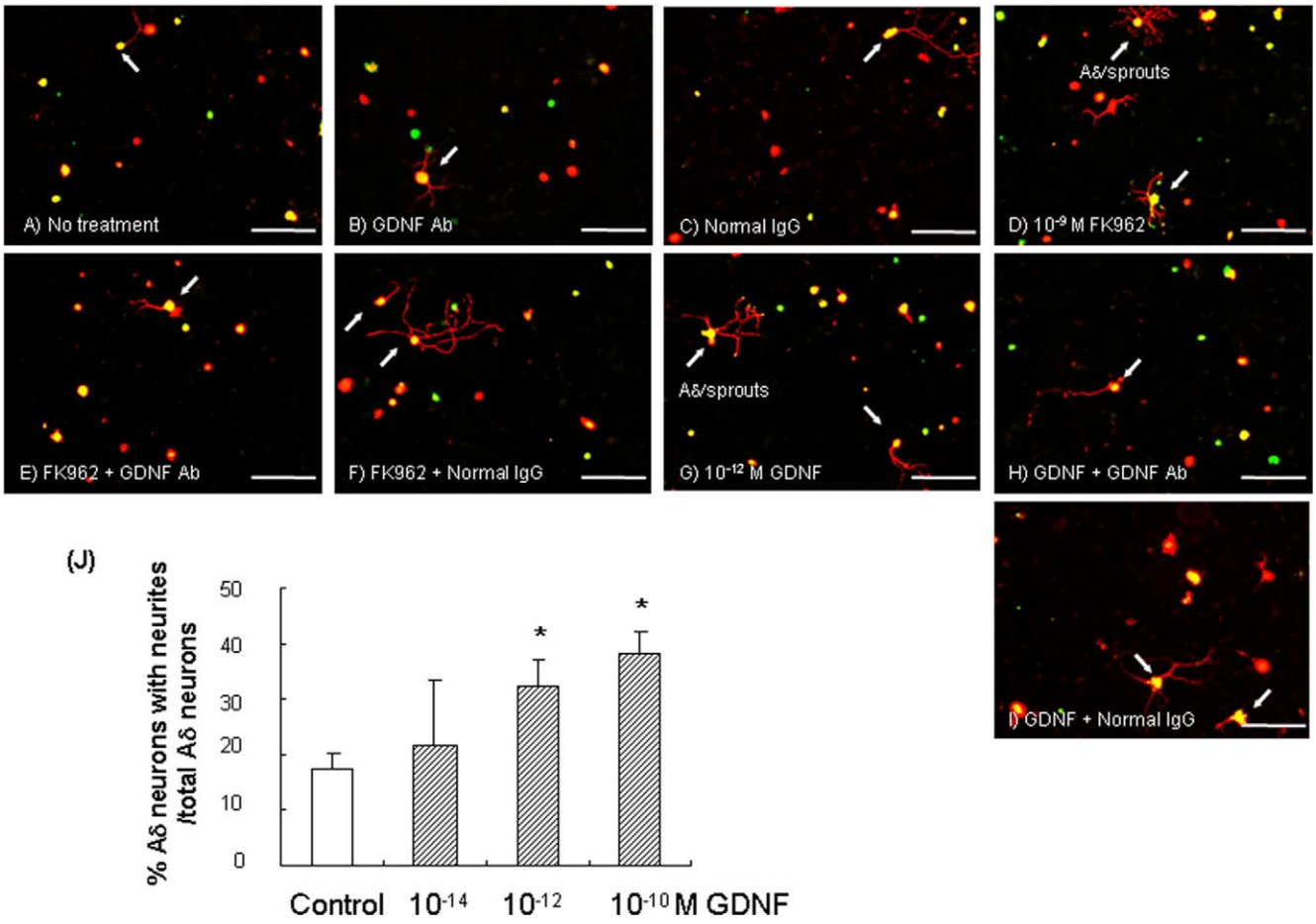

Figure 4. Merged immunofluorescence images obtained after double labeling with antibodies for substance P (*green*) and phospho-neurofilament H (*red*), showing Aδ neurons (*yellow*) and neurite elongation (*white arrows*) caused by FK962 and GDNF, and inhibition by antibody against GDNF. Panel labels indicate cells: (**A**) Without treatment. (**B**) Treatment with 1 μg/mL antibody against GDNF. (**C**) 1 μg/mL normal IgG. (**D**) 10^{-9} M FK962. (**E**) 10^{-9} M FK962 plus 1 μg/mL anti-GDNF antibody. (**F**) 10^{-9} M FK962 plus 1 μg/mL normal IgG. (**G**) 10^{-12} M GDNF, (**H**) 10^{-12} M GDNF plus 1 μg/mL anti-GDNF antibody. (**I**) 10^{-12} M GDNF with 1 μg/mL normal IgG. (**J**) Dose response curve for neurite elongation caused by culturing with increasing concentrations of exogenous GDNF. Scale bar = 200 μm. Data are means ± SD ($n = 3$). $^*P < 0.05$ relative to control using Dunnett's test.

(Fig. 3A, right) in the scratched area at time 0. After 24 hours without FK962 treatment, some neurites elongated into the scratched area (Fig. 3B, lower left, arrowheads). More elongated neurites were observed in the scratched area when the cells were treated with 10^{-9} M FK962 (Fig. 3B, lower right). The total length of these neurites was statistically increased compared with the nontreated controls (Fig. 3C).

Role of GDNF in FK962-Induced Neurite Elongation

TG cells cultured with exogenous GDNF showed Aδ neurons with more elongated neurites (Fig. 4G, white arrows) compared with nontreated controls (Fig. 4A), similar to the effect of FK962 (Fig. 4D). The dose response of GDNF on increased Aδ sprouting was linear, with 10^{-12} and 10^{-10} M GDNF showing statistical significance (Fig. 4J). GDNF antibody inhibited GDNF-induced (Fig. 4H) and FK962-induced (Fig. 4E) neurite elongation in Aδ neurons, while normal control IgG did not affect enhanced neurite elongation (Figs. 4F, 4I). Cell counting confirmed that coculture with GDNF antibody significantly reduced the number of Aδ neurons with elongated neurites after FK962 and exogenous GDNF treatments (Fig. 5).

Since GDNF is a putative positive effector of FK962-induced neurite elongation and regeneration, the expression of mRNAs for GDNF and its receptor were measured. In mixed TG cells, levels of mRNA for the GDNF were quite high compared with brain (Fig. 6A), and GDNF mRNA was present in isolated neurons and glial cells (Fig. 6B). Levels of mRNA for the GFRα1 subunit of the GDNF receptor complex were also high in isolated neurons compared with brain (Fig. 6C).

Negligible Direct Roles for NGF and SST in FK962-Induced Neurite Elongation

Exogenous NGF increased the number of Aδ neurons with elongated neurites in cultured TG cells (Fig. 7A). NGF antibody, but not normal control IgG, inhibited this NGF-induced neurite elongation in Aδ neurons. However, in contrast to GDNF antibody, FK962-induced neurite elongation was not reduced by treatment with NGF antibody (Fig. 7A).

Since FK962 was previously shown in brain to enhance the release of SST,[14] we also attempted to measure FK962 release

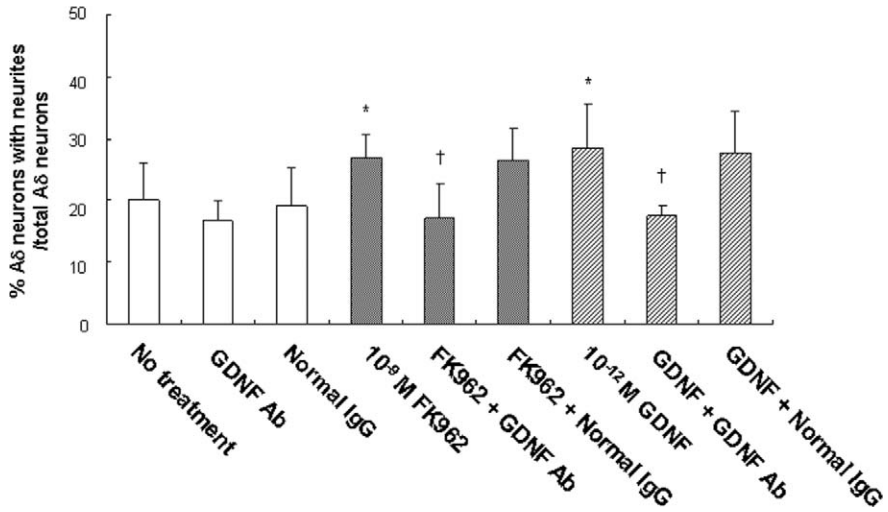

Figure 5. Analysis showing statistically significant reduction in FK962- or GDNF-induced Aδ neurite elongation by treatment with GDNF antibody. Data are means ± SD ($n = 8$). *$P < 0.05$ relative to no treatment, †$P < 0.05$ relative to 10^{-9} M FK962 or 10^{-12} M GDNF using Dunnett's test.

of SST by TG cells into the medium. SST protein was not detected by ELISA (2×10^{-11} M detection limit) in the medium (data not shown). Further, addition of 10^{-11} M SST to cultured TG cells did not induce neurite elongation; and high, nonphysiologic levels of 10^{-6} M SST only modestly enhanced elongation (Fig. 7B).

Discussion

The major finding of the present experiments was that GDNF, and not NGF or SST, played a direct role in FK962-induced neurite elongation/regeneration in cultured rat TG cells. We surmised that FK962 induces GDNF release from glial and/or neuronal cells, the GDNF is bound by the GDNF receptors on the TG axonal termini, and the GDNF is endocytosed and undergoes classical retrograde transport to initiate cell signaling for neurite expansion (Fig. 1A). This was also supported by findings that in isolated neurons from trigeminal ganglia, levels of mRNA for the GFRα1 subunit of the GDNF receptor complex were quite high compared with brain (Fig. 6C). The GDNF hetero-dimer receptor consists of a GFRα1 binding site for GDNF and a c-RET tyrosine kinase receptor site for transduction of downstream signals.[20]

The downstream signals activated by FK962 treatment were not defined in our TG cells, but an analog of FK962 stimulated activation of ERK1/2 and transcription of GDNF in c-Fos- and CREB-dependent mechanisms in cultured astrocytes.[11] The dipeptide leucine-isoleucine, a structural analog of FK506, activated the heat shock protein 90/Akt/CREB signaling pathway and stimulated GDNF expression in cultured neuronal hippocampus.[21] Similarly, valproic acid activated CREB and promoted GDNF expression in the rat hippocampus and frontal cortex.[22] In our cultured TG cells, FK962 may cause ERK activation and increase CREB-dependent transcription of GDNF. GDNF promotes survival and axonal regeneration in a wide variety of neuronal populations.[13] In the present experiment, antibody for GDNF prevented action of FK962 and GDNF, suggesting that GDNF was a factor for neurite elongation induced by FK962. Note that the GDNF protein levels secreted after FK962 treatment were probably quite low or localized since they were undetectable in our culture medium using a standard ELISA assay with a detection limit of 2×10^{-12} M GDNF.

Of course, FK962 may stimulate release of other factors besides GDNF to enhance TG neurite formation. For example, a previous report indicated that FK962 may enhance brain cognition by increasing secretion of SST.[14] Adding SST to

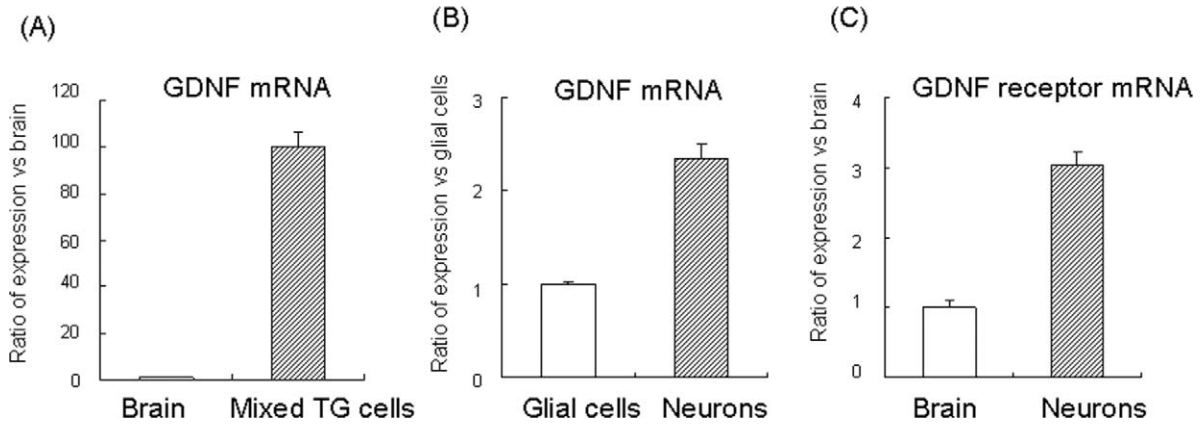

Figure 6. Expression of mRNA for GDNF in: (**A**) Brain and mixed TG cells and (**B**) glial cells and neurons; data are means ± SD ($n = 3$). (**C**) Expression of mRNA for GFRα1 in brain and isolated neurons; data are means ± SD ($n = 3$).

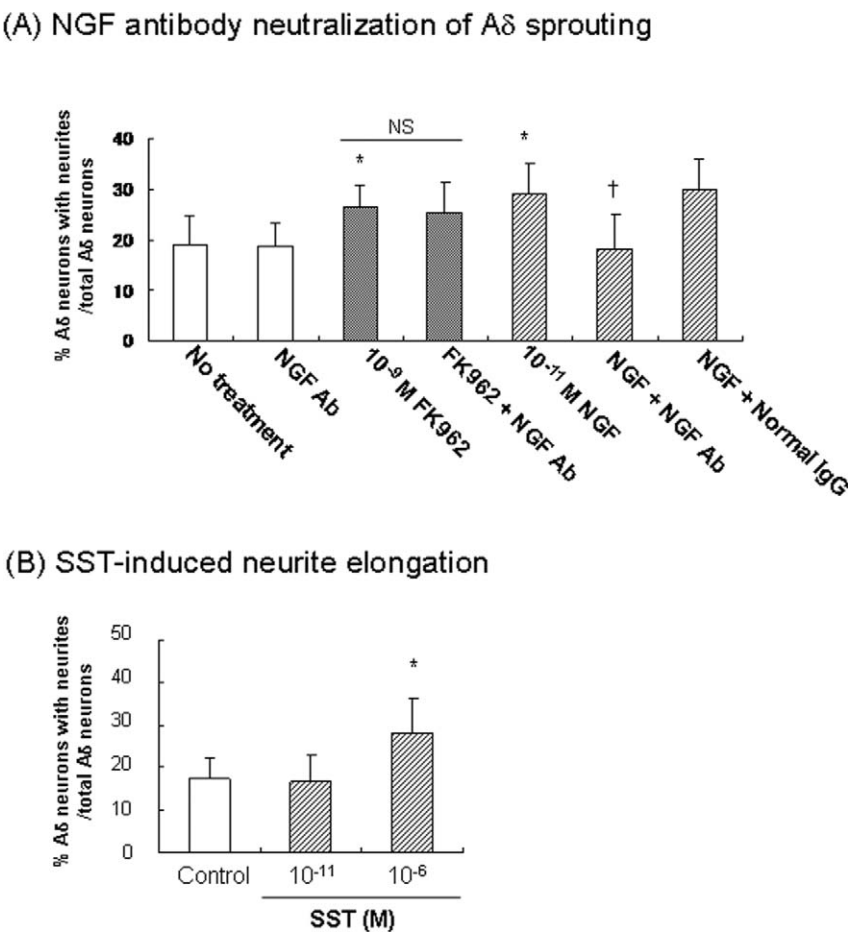

Figure 7. (**A**) Analysis of cell counts showing statistically significant reduction in NGF-induced, but not FK962-induced Aδ neurite elongation, by treatment with NGF antibody; data are means ± SD ($n = 5$). *$P < 0.05$ relative to no treatment; †$P < 0.05$ relative to 10^{-11} M NGF using Dunnett's test. NS, no significant difference relative to 10^{-9} M FK962 using Student's *t*-test. (**B**) Quantification of Aδ neurons with elongated neurites treated with exogenous SST; data are means ± SD ($n = 5$). *$P < 0.05$ relative to control using Dunnett's test.

PC12[23] and rat cerebellar granule cells[15] was also shown to induce neurite outgrowth. We thus expected FK962 to affect SST secretion in our cultured TG cells. However, the concentrations of SST in the medium after FK962 treatment were lower than 2×10^{-11} M (detection limit of ELISA), and addition of 10^{-11} M, SST to our culture system, did not induce neurite elongation (Fig. 7B), suggesting that SST was not a mechanism for FK962-induced neurite elongation. These data also suggested that different mechanisms might exist for neurite outgrowth in the peripheral TG and central nerve systems.

Previous studies showed that NGF had beneficial effects on the early recovery of corneal sensitivity after LASIK.[9] The present studies found that NGF caused neurite elongation in TG cells, and NGF antibody inhibited NGF-induced, but not FK962-induced neurite elongation (Fig. 7A). Thus, neither NGF nor SST seems to be a direct mediator of FK962-induced neurite elongation in TG cells. While it is too early to generalize, we did find it interesting that FK962-induced neurite elongation of TG cells may be dominated by GDNF signaling.

Our study supports the possibility of therapeutic use of FK962 in situations requiring elongation/regeneration of corneal neurons, such as in LASIK surgery. The sensory neurons innervating the cornea are located in the medial or ophthalmic region of the trigeminal nerve.[24] In cornea, fibers forming the basal epithelial plexus and branch into the epithelium originate from myelinated Aδ- and unmyelinated C-fibers.[25] Aδ fibers participate in mechanical sensitivity in cornea, which is very important for the blinking reflux leading to tear secretion.[26] Corneal sensory nerves, including Aδ fibers, are amputated during refractive surgery, such as in LASIK and photo-refractive keratectomy,[4] causing decreased corneal sensitivity and leading to dry eye. Our previous report showed that FK962 promoted neurite elongation in TG neurons and promoted recovery of corneal sensitivity during nerve regeneration in the rabbit.[7] We thus hypothesize that FK962 induces production of GDNF in TG cells, and the released GDNF promotes neurite elongation in Aδ fibers of the TG. This repair mechanism could form the basis for drug therapy designed to repair Aδ neuron ablation caused by LASIK surgery. In the present experiment, however, C-fibers were not studied, although C-fibers are present in the cornea along with Aδ neurons[6] and are implicated in dry eye.[27] Since neurite elongation occurred at similar levels in Aα/β/δ neurons and Aδ neurons (Fig. 2), FK962 may also induce neurite elongation in C-fibers. Future studies are required to specifically clarify neurite elongation of the C-fibers by FK962.

Acknowledgments

Thomas R. Shearer is a paid consultant for Senju Pharmaceutical Co., Ltd., a company that may have a commercial interest in the results of this research and technology. Mitsuyoshi Azuma is an

employee of Senju Pharmaceutical Co., Ltd. These potential conflicts of interest have been reviewed and managed by the OHSU.

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