Immunophilin Ligands and GDNF Enhance Neurite Branching or Elongation from Developing Dopamine Neurons in Culture

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Neurotrophic effects of immunophilin ligands have been shown in animal models of peripheral and central nervous system insult. To investigate the specific growth-promoting effects of these compounds, we examined the effects of various immunophilin ligands on primary dopamine (DA) neurons in culture and compared these with a well-known DA trophic factor, glial cell line-derived neurotrophic factor (GDNF). In neuronal cultures from Embryonic Day 14 ventral mesencephalon, enhanced *elongation* of DA neurites was observed with immunophilin ligands, which inhibited the phosphatase activity of calcineurin (FK506 and cyclosporin A) when compared to vehicle-treated cultures. This elongation was also observed with GDNF, known to exert its trophic effects through phosphorylation-dependent pathways. In contrast, immunophilin ligands that do not inhibit calcineurin (rapamycin and V-10,367) increased *branching* of DA neurites, suggesting that elongation is dependent upon maintained phosphorylation while branching is not. In addition, both V-10,367 and rapamycin antagonized the elongation effects of FK506 and induced branching. The antagonism of elongation (and reappearance of branching) illustrates the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. We show that the immunophilin FKBP12 (12-kDa FK506-binding protein) is expressed in ventral mesencephalic neuronal cultures and colocalizes with DA neurons. This work elucidates the specific growth-promoting effects by which GDNF and immunophilin ligands modify developmental growth processes of DA neurons, via their interactions with intracellular targets. © 2000 Academic Press

Key Words: **immunophilin; FKBP12; neurite; GDNF; calcineurin; dopamine cultures; FK506; rapamycin.**

INTRODUCTION

The regulation of neurite elongation and branching is fundamental for establishing neuronal circuitry (49, 59). Recently, immunosuppressive drugs such as FK506 and cyclosporin A (CsA) have been shown to enhance neurite outgrowth from various cell lines and peripheral nerve preparations (50, 67), as well as primary CNS neurons in culture (10). These compounds also protect against peripheral and central nervous system insult (10, 26, 65, 70), alter long term potentiation, (LTP) and long term depresssion, (LTD) (21, 73), increase the rate of peripheral nerve regeneration (25), and regulate neurotransmitter release (58, 69). The observed effects are partially or fully mediated by specific intracellular pathways that are affected by these ligands, but that remain unclear in the context of neuronal cell systems. In the immune system, the immunophilin ligand FK506 complexes with the immunophilin FKBP12 (12-kDa FK506-binding protein). This drug–immunophilin complex then binds to and inhibits the phosphatase activity of calcineurin (48), augmenting the phosphorylation of several substrates (12, 28, 42, 52, 64, 68), which leads to inhibition of cytokine synthesis and immunosuppression (20). Cyclosporin A also inhibits calcineurin, but through its interaction with the immunophilin cyclophilin B.

High levels of FKBP12 and cyclophilin in the brain (68) and their colocalization with calcineurin suggested that the neuronal trophic effects may also act through this intracellular pathway. The neuroprotective effects seen with immunophilin ligands in models of neurodegenerative disorders have led to the design of small-molecule ligands that bind to immunophilins, but are not immunosuppressive (do not interact with calcineurin (3)). One of these novel immunophilin ligands, V-10,367, has an affinity to FKBP12 similar to that of FK506 (K_i of 0.5 nM) and does not decrease the phosphatase activity of calcineurin (3). However, trophic effects are retained even in the absence of calcineurin inhibition: V-10,367 potentiates neuronal growth factor (NGF)-induced neurite outgrowth from immortalized cells in culture (26). In addition, neuroprotective and regenerative effects of these novel immunophilin ligands have been obtained in animal models of peripheral and central nervous system insult (10, 26, 70). We

TABLE 1

Immunophilin Ligands

Compound	Immunophilin interaction	Inhibition of calcineurin
FK506	FKBP12	Yes
CsA	Cyclophilin	Yes
Rapamycin	FKBP12	No
V-10,367	FKBP12	No

recently demonstrated neurotrophic effects of V-10,367 in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (10). Striatal dopaminergic (DA) innervation was spared from MPTP-induced degeneration in animals orally treated with V-10,367, while treatment with FK506 did not cause this effect. These studies suggest that inhibition of this phosphatase may not be required for the trophic effects obtained with these ligands in the nervous system.

To determine the specific effects of these compounds on the DA system, we evaluated the growth-promoting effects of these molecules on primary ventral mesencephalic (VM) DA neurons in culture. Comparisons were made between immunophilin ligands that interact with different intracellular targets and the trophic effects of glial cell line-derived neurotrophic factor (GDNF).

MATERIALS AND METHODS

Primary Ventral Mesencephalic Cultures

Primary cultures of DA neurons (previously shown to be 95% neuronal) were obtained from E14 Sprague–Dawley rat (Charles River, MA) ventral mesencephalon (VM) as described previously (10, 11). Briefly, tissue was dissociated by incubation in 0.025% trypsin solution (37°C, 15 min; Sigma) and triturated in a solution of DNAse (0.01%; Sigma) and trypsin inhibitor (0.05%; Sigma). Isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY) containing heat-inactivated horse serum (10%), glucose (6.0 mg/ml), penicillin (10,000 U/ml), streptomycin (10 mg/ml; Sigma), and glutamine (2 mM; Gibco). Five hundred microliters of suspension containing 5×10^5 cells/ml was plated into each well of 24-well trays (Falcon), precoated with poly-L-lysine (Sigma), containing 500 μl of serum-containing (S+) medium. Unattached cells were aspirated after 1 h, and 1 ml of fresh S+ medium containing immunophilin ligands (Table 1), GDNF, or vehicle (DMSO diluted at equivalent concentrations (1:1000) per well) was added. At 1 day in culture, the medium was replaced with defined medium (containing N2 cocktail; Gibco) containing immunophilin ligands or GDNF (using 30 kDa as the molecular weight of GDNF, the doses of 0.001–100 ng/ml convert to 33 pM–3.3 nM). At 2 days *in vitro* (DIV), cultures were fixed for 1 h with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS).

Immunohistochemistry

Cells were incubated in primary antibody against tyrosine hydroxylase (TH; 1:500; Pel-Freez, Rogers, AK) for 48 h at 4°C; antibody binding was visualized with reaction in 0.05% 3,3′-diaminobenzidine (DAB, Sigma). To study the expression of the immunophilin FKBP12 within VM neurons, cultures were double-labeled with antibodies against TH and FKBP12 (1:2000; Pharmingen, CA) and processed for immunocytochemical detection with texas red- and fluorescein-conjugated secondary antibody (1:200; Jackson ImmunoResearch; West Grove, PA). Fluorescent images of double-labeled FKBP12 and TH was visualized using a confocal argon laser scanning microscope (Leika), processed with Leika Physiology Module Software.

Quantification of Neuron Survival and Neurite Outgrowth

For neuron survival, four random fields at each corner of each well were selected for cell counts, as previously described (10): at 200× magnification, and using an eyepiece grid, the number of cells in each 200×20-μm corner and center of the grid was counted (5 counts per field). Counts consisted of the total number of neurons in each grid region for neuronal viability (defined morphologically in phase contrast and confirmed by trypan blue exclusion test), as well as the number of TH+ neurons. For analysis of neurite elongation and branching, two fields from opposite sides of each well were captured in Adobe Photoshop, and 10 randomly chosen TH+ neurons per field were analyzed. Each neurite from a TH+ neuron was traced and measured (in millimeters) to determine *elongation* effects, and the number of neurites extending from a single TH+ neuron was counted to determine *branching* effects (Fig. 1). The measurements from the 10 TH+ neurons per field were averaged to obtain two measurements per well. Each dose of each compound was run in duplicate per experiment, and data are expressed as percentage control from two to four pooled experiments (each dose in duplicate per experiment, for $n = 8$–16 per dose). All comparisons were evaluated using analysis of variance (ANOVA) in JMP Version 3.1 (SAS Institute, NC). When significance was obtained, post-hoc Tukey–Kramer HSD was performed to compare significant differences between groups (*$P < 0.05$). Error bars represent SEM.
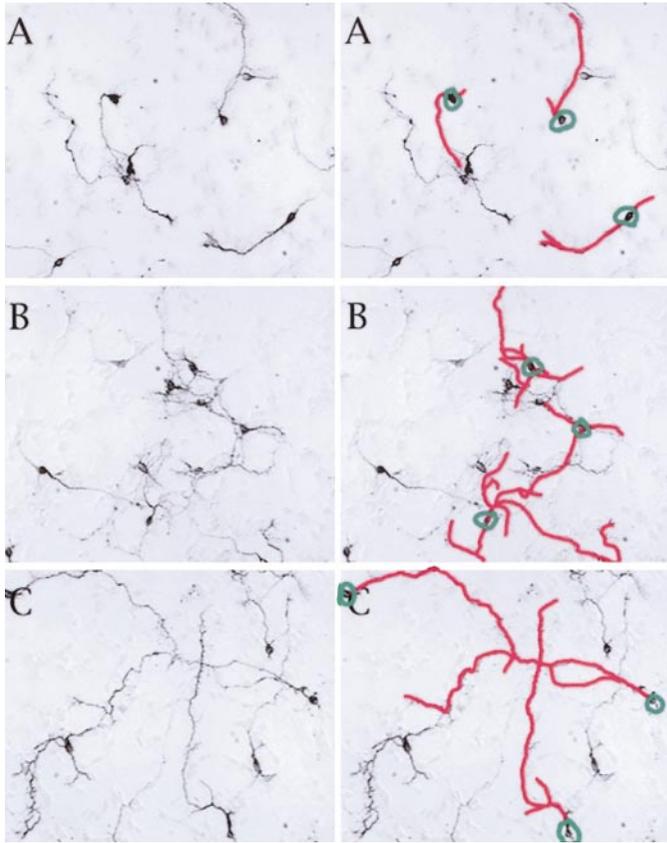
FIG. 1. Measurement of distinct effects of immunophilin ligands on neurite outgrowth of primary DA neurons. Parallel panels illustrate the method of measuring DA neurite outgrowth (stained for TH) in three representative cells (see Materials and Methods, and as quantified in Fig. 2): each TH+ neurite (outlined in red) was traced, counted, and measured from TH+ cell bodies (circled in green). (A) Neurite outgrowth of DA neurons from untreated primary cultures of E14 VM after 2 days in culture. (B) Enhanced *branching* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μM V-10,367. (C) Enhanced *elongation* of TH+ neurites from DA neurons after 2-day treatment with 1.0 μM FK506.

RESULTS

To better define intracellular targets and mechanisms of the trophic effects observed with immunophilin ligands, we utilized a system of primary DA neurons from E14 VM. By immunostaining VM cultures for TH (used here as a marker for DA neurons, Fig. 1) we investigated the effects of immunophilin ligands (Table 1) on neurite outgrowth. After 2 days in culture, the vehicle-treated primary DA neurons developed fairly short, unbranched TH+ neurites (Fig. 1A). Treatment of cultures for 2 days with the immunophilin ligand V-10,367 produced a significantly larger number of neurites from each TH+ neuron (Fig. 1B), whereas treatment with FK506 increased the length of neurites from TH+ neurons (Fig. 1C). We quantified these effects as previously described (10). Briefly, each neurite from a TH+ neuron was traced and measured (in millimeters) to determine its length (elongation), and the number of neurites extending from each TH+ neuron was counted (branching). To determine effects of immunophilin ligands or GDNF on neuronal viability, the total numbers of surviving neurons were analyzed: quantification revealed no effect of treatment with any of the immunophilin ligands or GDNF on total number of neurons (mean number of neurons per field = 128.75; $P > 0.768$) or TH+ neurons (mean number of TH+ neurons per field = 8.31; $P > 0.258$) after 2 days in culture.

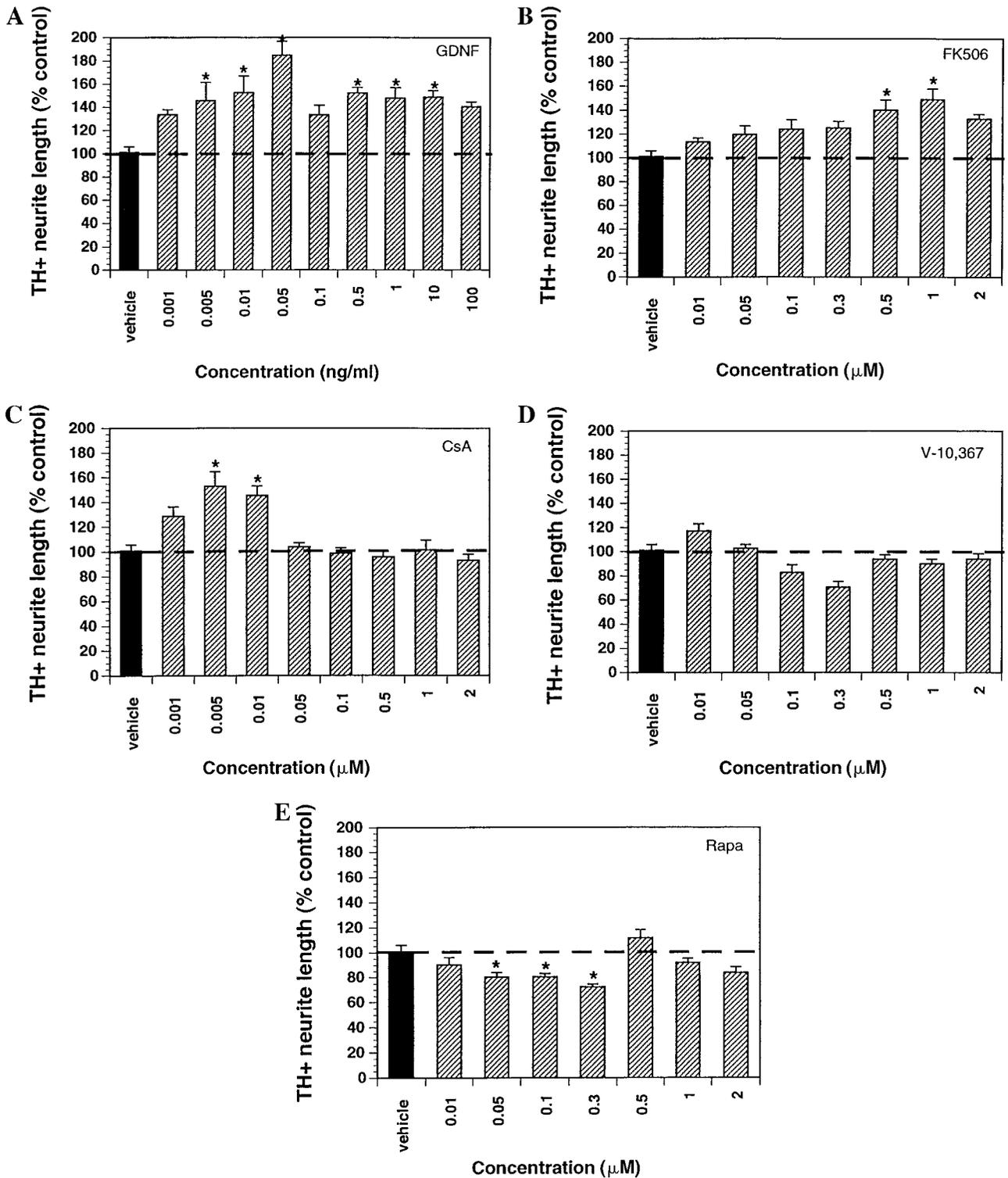### Elongation of Developing DA Neurites

To compare the trophic effects of immunophilin ligands to a well-known trophic factor for DA neurons, we first determined the specific effects of GDNF on neurite development. Neurite outgrowth was significantly influenced by 2-day treatment with GDNF (Fig. 2A): the average length of TH+ neurites was significantly longer than that of those in vehicle-treated cultures at a range of doses. We then compared this effect with two immunophilin ligands; FK506 (Fig. 2B) and CsA (Fig. 2C) that bind their respective immunophilins and subsequently inhibit calcineurin. Both FK506 and CsA significantly enhanced elongation of neurites from TH+ neurons. We then analyzed two immunophilin ligands that do not inhibit calcineurin: V-10,367 (Fig. 2D) and rapamycin (Fig. 2E). These compounds had no effect on the length of TH+ neurites. Interestingly, rapamycin showed a significant decrease in the length of neurites at some doses (Fig. 2E).
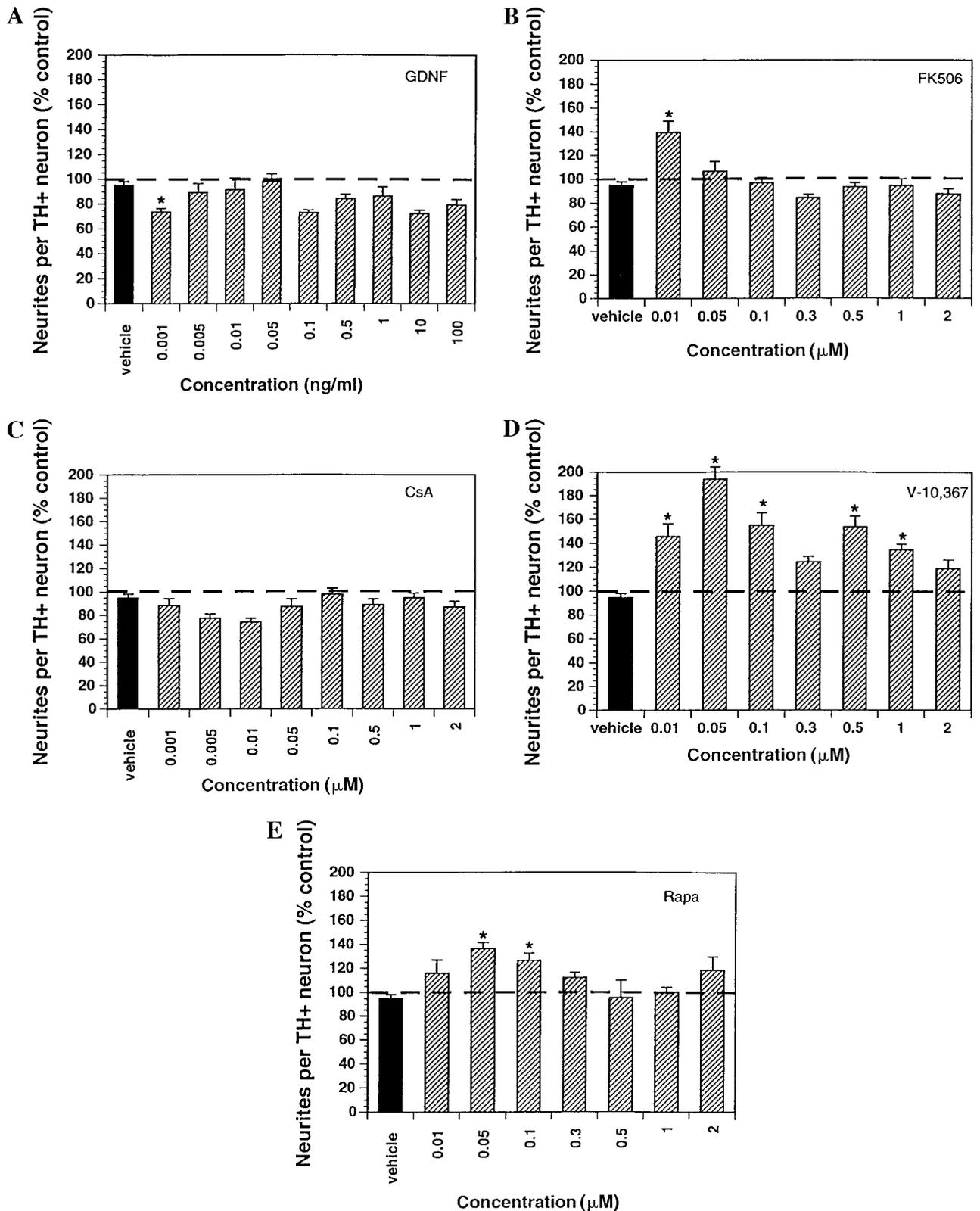
Branching of Developing DA Neurites

We next determined whether GDNF could affect the number of neurites extending from primary DA neurons. In contrast to elongation, neurite branching was not enhanced with GDNF (Fig. 3A). On the contrary, there tended to be fewer neurites per TH+ neuron than in vehicle-treated cultures at one dose. FK506 did not enhance branching at the doses that caused elongation of neurites (Fig. 3B); however, at one low dose (0.01 μM), a higher number of neurites was observed. The immunophilin ligand CsA had no effect on branching (Fig. 3C). In contrast, the two compounds that do not inhibit calcineurin (V-10,367 and rapamycin) showed significantly enhanced branching of TH+ neurites (Figs. 3D and 3E, respectively). The most robust effect with V-10,367 was observed at 0.05 μM, when DA neurons developed 2× the number of TH+ neurites that the vehicle-treated cultures developed.

Relationship between Branching and Elongation of Developing DA Neurites

In all cases where a compound produced a significant effect on DA neurite development (as previously ob-

FIG. 2. Elongation of developing DA neurites. Length of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF produced significantly longer neurites from TH+ neurons (A), as did FK506 (B) and CsA (C). The immunophilin ligand V-10,367, which does not inhibit the phosphatase calcineurin, showed no change in length of TH+ neurites (D), while rapamycin, which also does not inhibit calcineurin, showed a significant decrease in length of TH+ neurites (E). Tukey–Kramer HSD, *$P < 0.05$; error bars represent SEM.

FIG. 3. Branching of developing DA neurites. Number of TH+ neurites (percentage control) from primary cultures of E14 VM after 2 days of treatment. GDNF showed a decrease in branching of TH+ neurites only at the lowest dose (A), while FK506 showed a slight branching effect at its lowest dose (B). CsA showed no effect on branching of TH+ neurites (C). V-10,367 (D) and rapamycin (E), compounds that do not inhibit the phosphatase calcineurin, both showed significantly enhanced branching of TH+ neurites. Tukey–Kramer HSD, *$P < 0.05$; error bars represent SEM.

served with classic neurotrophic factors (36)), a bell-shaped dose–response curve was apparent (see Figs. 2 and 3). In several cases when neurons showed enhanced elongation they did not simultaneously show increased branching, and vice versa. For instance, two doses of rapamycin (0.05 and 0.1 μM) significantly enhanced branching (Fig. 3E), whereas these doses showed significantly shorter TH+ neurites (Fig. 2E). Conversely the elongation produced by 0.005 and 0.01 μM CsA (Fig. 2C) was paralleled by a trend toward decreased branching at these doses (Fig. 3C). Similarly, the only dose of FK506 that did induce branching (0.01 μM, Fig. 3B) showed the lowest value of neurite length (Fig. 2B).

Pharmacological Targets of Trophic Effects

To further define the intracellular mechanisms involved in the elongation and branching observed, we determined the presence of the immunophilin FKBP12 in VM cultures. FKBP12 was expressed in these neurons after 2 days in culture (Fig. 4A) and colocalized with TH+ neurons (Figs. 4B and 4C). We used combinations of V-10,367 or rapamycin with FK506 to determine whether the elongation effect of FK506 involved signaling through calcineurin via its interaction with FKBP12. Rapamycin and V-10,367 also bind FKBP12 and compete with FK506 for FKBP12 binding sites. FK506 (1.0 μM) did not elongate TH+ neurites in the presence of V-10,367 or rapamycin (0.5 and 1.0 μM, Fig. 4D). This effect was observed with 0.5 μM FK506 as well (data not shown). However, V-10,367 and rapamycin were able to induce branching in the presence of FK506 (Fig. 4E).

DISCUSSION

### Distinct Growth-Promoting Effects of Immunophilin Ligands

In the present study, we show that immunophilin ligands can enhance branching or elongation of neurite outgrowth from DA neurons. The identification of distinct aspects of neurite development allowed us to pharmacologically dissect specific effects of various immunophilin ligands and compare their effects with those of GDNF. The distinction between branching and elongation of neurites has previously been demonstrated when developing DA neurons are grown in the presence of growth factors (1, 5, 71) or their target striatal cells (14, 34, 62). For instance, exposure of VM neurons to conditioned medium from VM induces growth of dendrite-like neurites (short with a high number of branches), while striatal-conditioned medium stimulates growth of axon-like neurites (62). Though we did not differentiate between axons and dendrites (90% of neurites were MAP+, making selec-

tive analysis unreliable, Costantini et al., unpublished observations), independent regulation of axonal and dendritic initiation and elongation from DA neurons may play a role in the effects observed here. Studies have shown GDNF-induced neurite outgrowth from peripheral (16, 44) and VM neurons in culture (18, 36, 47) or after transplantation of VM tissue into adult brain (2, 29, 40, 61, 63, 80). Though the most prominent influence of GDNF upon developing DA neurons is enhanced survival (17, 43, 46), we did not observe this effect, most likely due to the short time course of our studies.

Several studies have observed enhanced neurite outgrowth with immunophilin ligands that do not inhibit calcineurin (26, 67); however these studies utilized transformed cell lines (dependent on NGF for outgrowth) and DRG explants and did not quantify differences between elongation and branching. The primary DA neuron cultures used in the present study were chosen as a more physiologic system for analyzing neurite outgrowth than growth factor-responsive cell lines or peripheral culture systems.

Generally, growth-promoting effects of neurotrophic factors depend on receptor activation. The primary intracellular receptors for the immunophilin ligands are FKBPs and cyclophilins, immunophilins that are highly expressed in the adult rat brain (13, 68). The expression of FKBP12 in fetal VM cultures shown here suggests a physiological role for this protein in developing DA neurons. The observed expression of FKBP12 in all cells in VM cultures suggests that the immunophilin ligands may also affect the GABA neurons present in these cultures. However, these GABA neurons do not extend neurites to the same extent as DA neurons after 2 days in culture. GABA markers stained approximately 90% of the cells and neurites, making analysis of neurites from individual cells unreliable. No obvious changes in neurite outgrowth were observed in this neuronal population after treatment with immunophilin ligands or GDNF (Costantini et al., unpublished observations). Alternatively, the immunophilin ligands may be acting through other immunophilins such as FKBP13 (77), FKBP25 (22), or FKBP52 (24, 72).

The receptor for CsA is the immunophilin cyclophilin (31), also expressed in the substantia nigra (13). Though CsA shows an affinity for its immunophilin that is 10-fold lower than FK506 has for FKBP12 (67), we observed enhanced elongation at lower doses with CsA than with FK506. This suggests that pathways other than the CsA/cyclophilin interaction may be involved. In contrast to our results, CsA prevented axonal elongation in cultured cerebellar neurons with no effect on neurite formation (19) and did not increase axonal regeneration in the sciatic nerve model (78), suggesting that cyclophilin-mediated trophic effects may not be active in these specific neuronal systems.
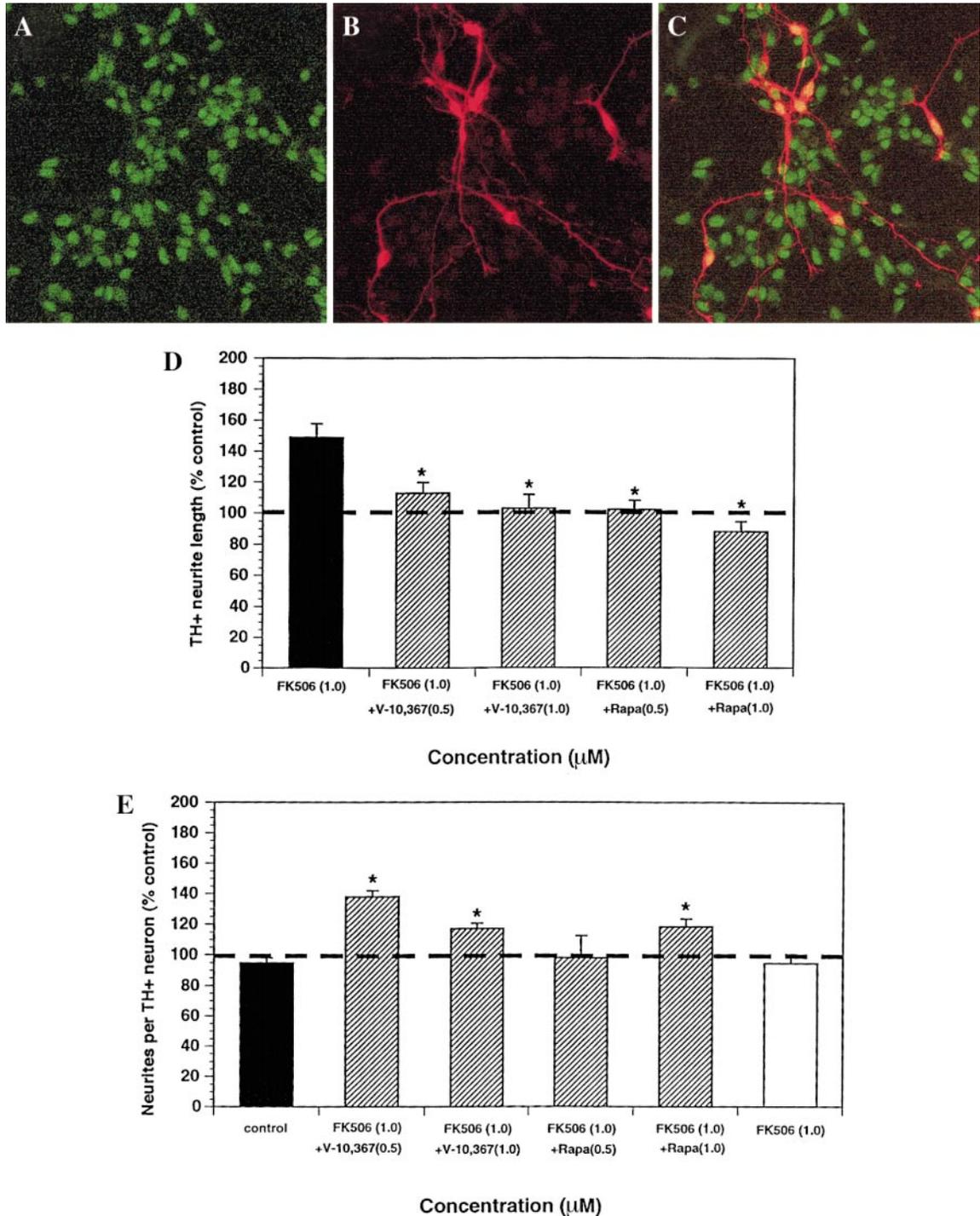
FIG. 4. Intracellular interactions of immunophilin ligands. (A) The immunophilin FKBP12 (green) is expressed in all neurons of primary cultures from E14 VM at 2 DIV. (B) The same field showing TH+ neurons (red). (C) Colocalization of FKBP12 and TH+ neurons (yellow). (D) FK506-induced elongation can be inhibited by compounds that compete for FKBP12-binding sites, such as V-10,367 and rapamycin. (E) Branching can occur with V-10,367 and rapamycin in the presence of FK506. Tukey–Kramer HSD, *$P < 0.05$; error bars represent SEM.

The bell-shaped immunophilin ligand dose–response curves in the present study suggest an optimal immunophilin ligand concentration for maximal trophic effects. Such effects have previously been explained by the "set-point hypothesis" of both growth factors and Ca+ levels (41, 55), where optimal trophic effects are observed with a mid level of growth factor or Ca+ level and low or high levels produce suboptimal or even toxic effects. Consistent with our present results (though inherent variability may also be responsible for the bell

shapes of these curves), a bell-shaped dose–response curve has been observed for optimal trophic effects of GDNF and FK506 (36, 78). Neurite sprouting and elongation are promoted only when intracellular Ca^{2+} levels are within a permissive range (53–55, 60). Immunophilin ligands have been shown to regulate $Ca+$ levels via their interactions with two Ca^{2+} channels found in the brain, the ryanodine receptor (RyR), and the inositol triphosphate receptor (IP3R) (7, 8, 37, 38, 66). Furthermore, these receptors are expressed in the primary VM neurons used in the present studies (Costantini, unpublished observations).

Role of Phosphorylation in Elongation or Branching of DA Neurites

We observed enhanced elongation with immunophilin ligands that inhibit the phosphatase activity of calcineurin (48) through their binding to their respective immunophilins (31, 32), as well as with GDNF which requires phosphorylation for its trophic effects (35). Calcineurin is expressed in the substantia nigra (13, 27), and its inhibition maintains the phosphorylation levels of several substrates (12, 28, 42, 52, 64, 68) that are critically involved in neuronal outgrowth (51, 54). Moreover, inhibition of calcineurin by a FK506 analog (ascomycin) stabilized F-actin in cultured hippocampal neurons (30), suggesting a role for calcineurin in cytoskeletal systems during neurite elongation (28).

In our study, the elongation observed with ligands inhibiting calcineurin was also observed with GDNF. The GDNF receptor system consists of GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) and the tyrosine kinase Ret (39), both of which are expressed in developing VM (4, 18, 74, 79). Activation of Ret tyrosine kinase activity by GDNF results in formation of neuritogenesis-associated lamellipodia (75), and tyrosine kinase inhibitors suppress GDNF-induced neurite outgrowth in cell lines (35). Thus, the compounds in our study that showed enhanced elongation of DA neurites are known to maintain phosphorylation levels within the cell, either through kinase activation (GDNF) or through inhibition of calcineurin (FK506 and CsA). Assays determining phosphorylation states of growth-associated proteins, such as GAP43 (6), microtubule-associated proteins (45), nitric oxide synthase (12), actin depolymerization factor (56), and GTPases (23) are required to fully evaluate this hypothesis. Alternatively, the folding of cytoskeletal proteins, receptors, or ion channels (33) through the rotamase-inhibitory effects of immunophilin ligands may alter extension and branching of developing neurites (32, 33).

Given the enhanced branching of DA neurites observed with rapamycin and V-10,367, a mechanism independent of calcineurin inhibition for trophic effects must be in play. Other evidence supporting a calcineurin-independent trophic mechanism is the neuroprotection and regeneration observed with immunophilin ligands that do not inhibit calcineurin (10, 26, 67). We investigated the role of calcineurin in these growth-promoting effects by combining either V-10,367 or rapamycin with FK506. Rapamycin is a competitive inhibitor of FK506 due to its higher affinity for FKBP12 (15) (and we found that rapamycin produced its trophic effects on DA neurites at concentrations lower than those of FK506, reflecting a higher potency). Both rapamycin and V-10,367 antagonized the effects of FK506 on elongation, presumably by competing for FKBP12-binding sites and decreasing the ability of FK506 to inhibit calcineurin. At the lower doses of competing V-10,367 and rapamycin, FK506-induced elongation was blocked but no branching occurred. However, at the higher doses of V-10,367 and rapamycin, branching did occur, even in the presence of FK506. These results support the requirement for maintained phosphorylation to obtain enhanced elongation of DA neurites.

The antagonism of elongation (and reappearance of branching) elucidate the intrinsic abilities of developing DA neurons to *either* elongate or branch, but not both. During development, when DA axons reach their target, they stop elongating and begin to branch extensively (57). Growth cone motility is directly related to neurite branching and stabilizes outgrowth by holding elongation rate at a submaximal level (55). In this way, under conditions of neurite branching (as with V-10,367 and rapamycin), neurites would show reduced elongation.

In contrast to our observations, the enhancement of NGF-induced outgrowth from PC12 cells and DRG by FK506 was not blocked by rapamycin, yet rapamycin alone enhanced outgrowth in these systems (50, 70). In a separate study, FK506 and CsA *inhibited* the NGF-induced neurite extension and neuritogenesis from DRG, while rapamycin inhibited only extension (9). The intracellular pathways present in PC12 and DRG cells are different than those in the primary DA culture system used in the present study, in which we observe rapamycin-associated pathways contributing to increased branching of DA neurites.

In conclusion, this study provides insight into the growth-promoting effects of immunophilin ligands on the DA system. Dopaminergic neurons of the substantia nigra start elongating axons toward their striatal target soon after final differentiation around Embryonic Day 12; then these axons branch extensively to innervate the striatum (76). During this phase, the developing neuron (both in culture and in brain) is expressing and responding to specific proteins that affect neuritogenesis and elongation (6, 17). The contrasting effects we observed on branching and elongation by different immunophilin ligands and GDNF suggest that these molecules differentially modify the

activities, expression, or structural states of proteins involved in neurite outgrowth. These observations may be relevant to recent trophic effects observed *in vivo* with immunophilin ligands (10, 26, 67). Additional studies are required to further elucidate the biochemical and molecular pathways involved in these growth-promoting effects.

ACKNOWLEDGMENTS

REFERENCES

1. Aoyagi, A., K. Nishikawa, H. Saito, and K. Abe. 1994. Characterization of basic fibroblast growth factor-mediated acceleration of axonal branching in cultured rat hippocampal neurons. *Brain Res.* **661:** 117–126.

2. Apostilides, C., E. Sanford, M. Hong, and I. Mendez. 1998. Glial cell line-derived neurotrophic factor improves intrastriatal graft survival of stored dopaminergic cells. *Neuroscience* **83:** 363–372.

3. Armistead, D. M., M. C. Badia, D. D. Deininger, J. P. Duffy, J. O. Saunders, R. D. Tung, J. A. Thomson, M. T. DeCenzo, O. Futer, D. J. Livingston, M. A. Murcko, M. M. Yamashita, and M. A. Navia. 1995. Design, synthesis, and structure of non-macrocyclic inhibitors of FKBP12, the major binding protein for the immunosuppressant FK506. *Acta. Crystallogr.* **D51:** 522–528.

4. Baloh, R., M. Tansey, J. Golden, D. Creedon, R. Heuckeroth, C. Keck, D. Zimonjic, N. Popescu, E. Johnson, and H. Milbrandt. 1997. TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. *Neuron* **18:** 793–802.

5. Beck, K. D., B. Knusel, and F. Hefti. 1993. The nature of the trophic action of brain-derived neurotrophic factor, sed(1-3)-insulin-like growth factor-1, and basic fibroblast growth factor on mesencephalic dopaminergic neurons developing in culture. *Neuroscience* **52:** 855–866.

6. Benowitz, L. I., and A. Routtenberg. 1997. GAP-43: An intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* **20:** 84–91.

7. Brillantes, A. B., K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. C. Moschella, T. Jayaraman, M. Landers, B. E. Ehrlich, and A. R. Marks. 1994. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77:** 513–523.

8. Cameron, A. M., J. P. Steiner, D. M. Sabatini, A. I. Kaplin, L. D. Walensky, and S. H. Snyder. 1995. Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. *Proc. Natl. Acad. Sci. USA* **92:** 1784–1788.

9. Chang, H., K. Takei, A. Sydor, T. Born, F. Rusnak, and D. Jay. 1995. Asymmetric retraction of growth cone filopodia following focal inactivation of calcineurin. *Nature* **376:** 686–690.

10. Costantini, L., P. Chaturvedi, D. Armistead, P. McCaffrey, T. Deacon, and O. Isacson. 1998. A novel immunophilin ligand: Distinct branching effects on dopaminergic neurons in culture and neurotrophic actions after oral administration in an animal model of Parkinson's disease. *Neurobiol. Dis.* **5:** 97–106.

11. Costantini, L. C., L. Lin, and O. Isacson. 1997. Medial fetal ventral mesencephalon: A preferred source for dopamine neuron grafts. *Neuroreport* **8:** 2253–2257.

12. Dawson, T. M., J. P. Steiner, V. L. Dawson, J. L. Dinerman, G. R. Uhl, and S. H. Snyder. 1993. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate toxicity. *Proc. Natl. Acad. Sci. USA* **90:** 9808–9812.

13. Dawson, T. M., J. P. Steiner, W. E. Lyons, M. Fotuhi, M. Blue, and S. H. Snyder. 1994. The immunophilins, FK506 binding protein and cyclophilin, are discretely localized in the brain: Relationship to calcineurin. *Neuroscience* **62:** 569–580.

14. Denis-Donini, S., J. Glowinski, and A. Prochiantz. 1983. Specific influence of striatal target neurons on the in vitro outgrowth of mesencephalic dopaminergic neurites: A morphological quantitative study. *J. Neurochem.* **3:** 2292–2299.

15. Dumont, F., M. Melino, M. Staruch, S. Koprak, P. Fischer, and N. Sigal. 1990. The immunosuppressive macrolides FK506 and rapamycin act as reciprocal antagonists in murine T cells. *J. Immunol.* **144:** 1418–1424.

16. Ebendal, T., A. Tomac, B. Hoffer, and L. Olson. 1995. Glial cell line-derived neurotrophic factor stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia. *J. Neurosci. Res.* **40:** 276–284.

17. Engele, J. 1998. Spatial and temporal growth factor influences on developing midbrain dopaminergic neurons. *J. Neurosci. Res.* **53:** 405–414.

18. Feng, L., C. Wang, H. Jiang, C. Oho, K. Mizuno, M. Dugich-Djordjevic, and B. Lu. 1999. Differential effects of GDNF and BDNF on cultures ventral mesencephalic neurons. *Mol. Brain Res.* **66:** 62–70.

19. Ferreira, A., R. Kincaid, and K. Kosik. 1993. Calcineurin is associated with the cytoskeleton of cultured neurons and has a role in the acquisition of polarity. *Mol. Biol. Cell* **4:** 1225–1238.

20. Fruman, D., C. Klee, B. Bierer, and S. Burakoff. 1992. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and cyclosporin A. *Proc. Natl. Acad. Sci.* **89:** 3686–3690.

21. Funauchi, M., H. Haruta, and T. Tsumoto. 1994. Effects of an inhibitor for calcium/calmodulin-dependent protein phosphatase, calcineurin, on induction of long-term potentiation in rat visual cortex. *Neurosci. Res.* **19:** 269–278.

22. Galat, A., W. Lane, R. Standaert, and S. Schreiber. 1992. A rapamycin-selective 25-kDa immunophilin. *Biochemistry* **31:** 2427–2434.

23. Gallo, G., and P. Letourneau. 1998. Axon guidance: GTPases help axons reach their targets. *Curr. Biol.* **8:** R80–R82.

24. Gold, B., V. Densmore, W. Shou, M. Matzuk, and H. Gordon. 1999. Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J. Pharmacol. Exp. Ther.* **289:** 1202–1210.

25. Gold, B. G., K. Katoh, and T. Storm-Dickerson. 1995. The immunosuppressant FK506 increases the rate of axonal regeneration in rat sciatic nerve. *J. Neurosci.* **15:** 7509–7516.

26. Gold, B. G., M. Zeleny-Pooley, M. S. Wang, P. Chaturvedi, and D. M. Armistead. 1997. A non-immunosuppressive FKBP-12 ligand increases nerve regeneration. *Exp. Neurol.* **147:** 269–278.

27. Goto, S., Y. Matsukado, Y. Mihara, N. Inoue, and E. Miyamoto. 1986. The distribution of calcineurin in the rat brain by light and electron microscopic immunohistochemistry and enzyme-immunoassay. *Brain Res.* **397:** 161–172.

28. Goto, S., H. Yammamoto, K. Fukunaga, T. Iwasa, Y. Matsukado, and E. Miyamoto. 1985. Dephosphorylation of microtubule-associated protein 2, tau factor, and tubulin by calcineurin. *J. Neurochem.* **45:** 276–283.

29. Granholm, A., J. Mott, K. Bowenkamp, S. Eken, S. Henry, B. Hoffer, P. Lapchak, M. Palmer, C. van Horne, and G. Gerhardt. 1997. Glial cell line-derived neurotrophic factor improves sur-

vival of ventral mesencephalic grafts to the 6-hydroxydopamine lesioned striatum. *Exp. Brain Res.* **116:** 29–38.

30. Halpain, S., A. Hipolito, and L. Saffer. 1998. Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J. Neurosci.* **18:** 9835–9844.

31. Handschumacher, R., M. Harding, J. Rice, R. Drugge, and D. Speicher. 1984. Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* 544–547.

32. Harding, M. W., A. Galat, D. E. Uehling, and S. L. Schreiber. 1989. A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. *Nature* **341:** 758–760.

33. Helekar, S. A., D. Char, S. Neff, and J. Patrick. 1994. Prolyl isomerase requirement for the expression of functional homo-oligomeric ligand-gated ion channels. *Neuron* **12:** 179–189.

34. Hemmendinger, L. M., B. B. Garber, P. C. Hoffmann, and A. Heller. 1981. Target neuron-specific process formation by embryonic mesencephalic dopamine neurons in vitro. *Proc. Natl. Acad. Sci. USA* **78:** 1264–1268.

35. Hiwasa, T., K. Kondo, T. Hishiki, S. Koshizawa, K. Umezawa, and A. Nakagawara. 1997. GDNF-induced neurite formation was stimulated by protein kinase inhibitors and suppressed by Ras inhibitors. *Neurosci. Lett.* **238:** 115–118.

36. Hou, J. G., L. H. Lin, and C. Mytilineou. 1996. Glial cell-line derived neurotrophic factor exerts neurotrophic effects on dopaminergic neurons in vitro and promotes their survival and regrowth after damage by 1-methyl-4-phenylpyridinium. *J. Neurochem.* **66:** 74–82.

37. Jamil, G., R. Hagar, E. J. Kaftan, and B. E. Ehrlich. 1996. Effects of FK506 analogs on ryanodine receptors. *Biophys. J.* **70:** A75.

38. Jayaraman, T., A. Brillantes, A. P. Timerman, S. Fleischer, H. Erdjument-Bromage, P. Tempst, and A. R. Marks. 1992. FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.* **267:** 9474–9477.

39. Jing, S., D. Wen, Y. Yu, P. Holst, Y. Luo, M. Fang, R. Tamir, L. Antonia, Z. Hu, R. Cupples, J. Louis, S. Hu, B. Altrock, and G. Fox. 1996. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85:** 1113–1124.

40. Johansson, M., M. Friedemann, B. Hoffer, and I. Stromberg. 1995. Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. *Exp. Neurol.* **134:** 25–34.

41. Johnson, E. M., T. Koike, and J. Franklin. 1992. The "calcium set-point hypothesis" of neuronal dependence on neurotrophic factor. *Exp. Neurol.* **115:** 163–166.

42. King, M., C. Huang, P. Chock, A. Nairn, H. Hemmings, K. Chan, and P. Greengard. 1984. Mammalian brain phosphoproteins as substrates for calcineurin. *J. Biol. Chem.* **10:** 8080–8083.

43. Kreiglstein, K., C. Suter-Crazzolara, W. H. Fischer, and K. Unsicker. 1995. TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. *EMBO J.* **14:** 736–742.

44. Leclere, P., P. Ekstrom, A. Edstrom, J. Priestly, S. Averill, and D. Tonge. 1997. Effects of glial cell line-derived neurotrophic factor on axonal growth and apoptosis in adult mammalian sensory neurons in vitro. *Neuroscience* **82:** 545–558.

45. Letourneau, P. 1996. The cytoskeleton in nerve growth cone motility and axonal pathfinding. *Perspect. Dev. Neurobiol.* **4:** 111–123.

46. Lin, L., D. Doherty, J. Lile, S. Bektesh, and F. Collins. 1993. GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260:** 1130–1132.

47. Lin, L., T. Zhang, F. Collins, and L. Armes. 1994. Purification and initial characterization of rat B49 glial cell line-derived neurotrophic factor. *J. Neurochem.* **63:** 758–768.

48. Liu, J., J. Farmer, W. Lane, J. Friedman, I. Weissman, and S. Schreiber. 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66:** 807–815.

49. Lo, D. 1998. Instructive roles of neurotrophins in synaptic plasticity. *Prog. Brain Res.* **117:** 65–70.

50. Lyons, W. E., E. B. George, T. M. Dawson, J. P. Steiner, and S. H. Snyder. 1994. Immunosuppressant FK506 promotes neurites outgrowth in cultures of PC12 cells and sensory ganglia. *Proc. Natl. Acad. Sci. USA* **91:** 3191–3195.

51. Maness, P., H. Beggs, S. Klinz, and W. Morse. 1996. Selective neural cell adhesion molecule signalling by Src family tyrosine kinases and tyrosine phosphatases. *Perspect. Dev. Neurobiol.* **4:** 169–181.

52. Marcaida, G., E. Kosenko, M. D. Minana, S. Grisolia, and V. Felipo. 1996. Glutamate induces calcineurin-mediated dephosphorylation of Na+, K(+)-ATPase that results in its activation in cerebellar neurons in culture. *J. Neurochem.* **66:** 99–104.

53. Mattson, M. P. 1992. Calcium as sculptor and destroyer of neural circuitry. *Exp. Gerontol.* **27:** 29–49.

54. Mattson, M. P., P. B. Guthrie, and S. B. Kater. 1988. Components of neurite outgrowth that determine neuronal cytoarchitecture: Influence of calcium and the growth substrate. *J. Neurosci. Res.* **20:** 331–345.

55. Mattson, M. P., and S. B. Kater. 1987. Calcium regulation of neurite elongation and growth cone motility. *J. Neurosci.* **7:** 4034–4043.

56. Meberg, P., S. Ono, L. Minamide, M. Takahashi, and J. Bamburg. 1998. Actin depolymerization factor and cofilin phosphorylation dynamics: Response to signals that regulate neurite extension. *Cell Motil. Cytoskeleton* **39:** 172–190.

57. Newman-Gage, H., and A. Graybiel. 1988. Expression of calcium/calmodulin-dependent protein kinase in relation to dopamine islands and synaptic maturation in the cat striatum. *J. Neurosci.* **8:** 3360–3375.

58. Nichols, R., G. Suplick, and J. Brown. 1994. Calcineurin-mediated protein dephosphorylation in brain nerve terminals regulates release of glutamate. *J. Biol. Chem.* **269:** 23,817–23,823.

59. O'Leary, D., and S. Koester. 1993. Development of projection neuron types, axon pathways, and patterned connections of the mammalian cortex. *Neuron* **10:** 991–1006.

60. Rehder, V., and S. Kater. 1992. Regulation of neuronal growth cone filopodia by intracellular calcium. *J. Neurosci.* **12:** 1375–1386.

61. Rosenblad, C., A. Martinez-Serrano, and A. Bjorklund. 1996. Glial cell line-derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience* **75:** 979–985.

62. Rousselet, A., L. Felter, B. Chamak, and A. Prochiantz. 1988. Rat mesencephalic neurons in culture exhibit different morphological traits in the presence of media conditioned on mesencephalic or striatal astroglia. *Dev. Biol.* **129:** 495–504.

63. Sautter, J., J. Tseng, D. Braguglia, P. Aebischer, C. Spenger, R. Seiler, H. Widmer, and A. Zurn. 1998. Implants of polymer-encapsulated genetically modified cells releasing glial cell line-derived neurotrophic factor improve survival, growth, and function of fetal dopaminergic grafts. *Exp. Neurol.* **149:** 230–236.

64. Seki, K., H. C. Chen, and K. P. Huang. 1995. Dephosphorylation of protein kinase C substrates, neurogranin, neuromodulin, and MARCKS, by calcineurin and protein phosphatases 1 and 2A. *Arch. Biochem. Biophys.* **316:** 673–679.

65. Sharkey, J., and S. P. Butcher. 1994. Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature* **371:** 336–339.

66. Sharp, A., F. Nucifora, O. Blondel, C. Sheppard, C. Zhang, S. Snyder, J. Russell, D. Ryugo, and C. Ross. 1999. Differential cellular expression of isoforms of inositol 1,4,5-triphosphate receptors in neurons and glia in brain. *J. Comp. Neurol.* **406:** 207–220.

67. Steiner, J. P., M. A. Connelly, H. L. Valentine, G. S. Hamilton, T. M. Dawson, L. Hester, and S. H. Snyder. 1997. Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin, and cyclosporin A. *Nat. Med.* **3:** 421–428.

68. Steiner, J. P., T. M. Dawson, M. Fotuhi, C. E. Glatt, A. M. Snowman, N. Cohen, and S. H. Snyder. 1992. High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature* **358:** 584–586.

69. Steiner, J. P., T. M. Dawson, M. Fotuhi, and S. H. Snyder. 1996. Immunophilin regulation of neurotransmitter release. *Mol. Sci.* **2:** 325–333.

70. Steiner, J. P., G. S. Hamilton, D. T. Ross, H. L. Valentine, H. Guo, M. A. Connelly, S. Liang, C. Ramsey, J. J. Li, W. Huang, P. Howorth, R. Soni, M. Fuller, H. Sauer, A. C. Nowotnik, and P. D. Suzdak. 1997. Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. *Proc. Natl. Acad. Sci. USA* **94:** 2019–2024.

71. Studer, L., C. Spenger, R. W. Seiler, C. A. Altar, R. M. Lindsay, and C. Hyman. 1995. Comparison of the effects of the neurotrophins on the morphological structure of dopaminergic neurons in cultures of rat substantia nigra. *Eur. J. Neurosci.* **7:** 223–233.

72. Tai, P., M. Albers, H. Chang, L. Faber, and S. Schreiber. 1992. Association of a 59-kDa immunophilin with the glucocorticoid receptor complex. *Science* **256:** 1315–1318.

73. Torii, N., T. Kamashita, Y. Otsu, and T. Tsumoto. 1995. An inhibitor of calcineurin, FK506, blocks induction of long-term depression in rat visual cortex. *Neuroscience* **185:** 1–4.

74. Treanor, J., L. Goodman, F. deSauvage, D. Stone, K. Poulson, C. Bexk, C. Gray, M. Armanini, R. Pollock, F. Hefti, H. Phillips, A. Goddard, M. Moore, A. Buj-Bello, A. Davies, N. Asai, M. Takahashi, R. Vandlen, C. Henderson, and A. Rosenthal. 1996. Characterization of a multicomponent receptor for GDNF. *Nature* **382:** 80–83.

75. van Weering, D., and J. Bos. 1997. Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation. *J. Biol. Chem.* **272:** 249–254.

76. Voorn, P., A. Kalsbeek, B. Jorritsma-Byham, and H. Groenewegen. 1988. The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25:** 857–887.

77. Walensky, L., P. Gascard, M. Fields, S. Blackshaw, J. Conboy, N. Mohandas, and S. Snyder. 1998. The 13-kD FK506 binding protein, FKBP13, interacts with a novel homologue of the erythrocyte membrane cytoskeletal protein 4.1. *J. Cell Biol.* **141:** 143–153.

78. Wang, M. S., M. Zeleny-Pooley, and B. G. Gold. 1997. Comparative dose-dependence study of FK506 and cyclosporin A on the rate of axonal regeneration in the rat sciatic nerve. *J. Pharmacol. Exp. Ther.* **282:** 1084–1093.

79. Widenfalk, J., C. Nosrat, A. Tomac, H. Westphal, B. Hoffer, and L. Olson. 1997. Neurturin and glial cell line-derived neurotrophic factor receptor beta, novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J. Neurosci.* **17:** 8506–8519.

80. Wilby, M., S. Sinclaie, E. Muir, R. Zietlow, K. Adcock, P. Horellou, J. Rogers, S. Dunnett, and J. Fawcett. 1999. A glial cell line-derived neurotrophic factor-secreting clone of the Schwann cell line SCTM41 enhances survival and fiber outgrowth from embryonic nigral neurons grafted to the striatum and to the lesioned substantia nigra. *J. Neurosci.* **19:** 2301–2312.