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# Neurotrophins and Netrins Require Calcineurin/NFAT Signaling to Stimulate Outgrowth of Embryonic Axons

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## Summary

**Axon outgrowth is the first step in the formation of neuronal connections, but the pathways that regulate axon extension are still poorly understood. We find that mice deficient in calcineurin-NFAT signaling have dramatic defects in axonal outgrowth, yet have little or no defect in neuronal differentiation or survival. In vitro, sensory and commissural neurons lacking calcineurin function or NFATc2, c3, and c4 are unable to respond to neurotrophins or netrin-1 with efficient axonal outgrowth. Neurotrophins and netrins stimulate calcineurin-dependent nuclear localization of NFATc4 and activation of NFAT-mediated gene transcription in cultured primary neurons. These data indicate that the ability of these embryonic axons to respond to growth factors with rapid outgrowth requires activation of calcineurin/NFAT signaling by these factors. The precise parsing of signals for elongation turning and survival could allow independent control of these processes during development.**

## Introduction

The complex yet stereotyped morphologies of neurons arise during embryonic development through the growth of axons and dendrites from neuronal cell bodies. Extrinsic and intrinsic factors contribute to shaping these extensions (Edlund and Jessell, 1999; Gao et al., 1999). A variety of extracellular cues, including netrins and neurotrophins, stimulate, inhibit, and guide process extension and branching by binding receptors present on axonal and dendritic growth cones and along the axonal and dendritic shafts (Giger and Kolodkin, 2001; Huang and Reichardt, 2001; Tessier-Lavigne and Goodman, 1996). How neurites respond to these cues is determined by developmental programs that control the repertoire of expressed receptors and signal transduction molecules.

There is mounting evidence for dedicated transcriptional programs, acting after the initial specification of neurons into generic classes that regulate later aspects of their development, including the choice of pathway by their axons or the shape of their dendritic arbor. In the case of spinal motoneurons, initial specification occurs through the action of homeodomain and basic-helix-loop-helix transcription factors (Anderson, 2001; Briscoe et al., 2000; Ericson et al., 1992; Jessell, 2000; Shirasaki and Pfaff, 2002), but their subsequent choice of major axonal pathways (correlating with their columnar identity in the spinal cord) is directed by distinct combinations of LIM homeodomain transcription factors (Kania et al., 2000; Sharma et al., 1998; Thor et al., 1999; Tsuchida et al., 1994). Another example of transcription factors regulating later aspects of neuronal morphogenesis is provided by the homeodomain transcription factor Otx1, which is required for regulating stereotyped pruning of layer 5 cortical neuron branches (Weimann et al., 1999). Genetic screens in *Drosophila* have also identified the zinc finger protein Sequoia as an important regulator of dendrite development (Brenman et al., 2001; Gao et al., 1999) and the zinc finger protein Brakeless as critical for axon targeting during visual system development (Rao et al., 2000; Senti et al., 2000). Although some of these transcriptional processes may be activated autonomously in neurons as a consequence of an early specification event, in other cases their action may be regulated by late environmental signals. Coupling neuronal transcription to extracellular signals would allow fine tuning the timing of their activation. For example, expression of various ETS family transcription factors, which appear to control late aspects of neuronal morphogenesis (Arber et al., 2000; Livet et al., 2002), is regulated by the receipt of target-derived signals (Haase et al., 2002; Lin et al., 1998). Thus, the emerging picture is that specific aspects of neuronal morphogenesis may be controlled by dedicated transcriptional programs, some of which are regulated by environmental cues. However, the range of neuronal properties that are governed by changes in gene expression and the identity of key transcriptional regulators of such events remain largely unknown.

NFAT transcription complexes are appealing candidates for regulating aspects of neuronal morphogenesis because they integrate extracellular signals (Crabtree, 1989). Cell membrane signaling results in the assembly of NFAT transcription complexes in the nucleus and the activation of genes that are dependent on the cell type in which the signal is received (Crabtree and Olson, 2002; Graef et al., 2001b, 1999; Shaw et al., 1988). A rise in intracellular $Ca^{2+}$ activates the serine/threonine phosphatase calcineurin (Klee et al., 1979) and rapidly dephosphorylates the four cytoplasmic subunits NFATc1-4 (http://www.gene.ucl.ac.uk/nomenclature/genefamily/NFAT/NFAT.shtml) (Clipstone and Crabtree, 1992; Flanagan et al., 1991). Dephosphorylation of serines in the amino-termini of NFATc proteins by calcineurin exposes nuclear localization sequences leading to their rapid nuclear import. NFATc cytoplasmic subunits require other transcription factors for DNA binding, including AP-1, MEF2, GATA4, and additional factors generically referred to as nuclear partners (NFATn) (Flan-

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agan et al., 1991). The nuclear components of NFAT transcription complexes are often regulated by the PKC and Ras/MAPK pathways (Flanagan et al., 1991). Hence, the assembly of NFAT transcription complexes requires that $Ca^{2+}$/calcineurin signaling be coincident with other signals (Crabtree, 1989). Nuclear import of NFATc family members is opposed by rapid export induced by rephosphorylation mediated by the sequential actions of PKA and GSK3 (Beals et al., 1997). The rapid export of NFATc proteins from the nucleus can make NFAT signaling responsive to receptor occupancy and/or $Ca^{2+}$ channel dynamics (Dolmetsch et al., 1997; Graef et al., 1999; Timmerman et al., 1996).

Here, we provide evidence for an unexpected role for calcineurin and NFATc family members in controlling the outgrowth of embryonic axons both in vivo and in vitro. Our results suggest that calcineurin/NFAT signaling is required specifically for axon outgrowth stimulated by growth factors like neurotrophins and netrins and provides a potential regulatory site for controlling axonal elongation independent of neuronal survival.

## Results

### Mice Bearing Mutations in *NFATc2*, *c3*, and *c4* Have Defects in Axon Outgrowth

Profound defects in sensory axon projections were observed in embryos with combined deletions of either *NFATc3* and *NFATc4* (c3/c4 mutants) or of *NFATc2*, *NFATc3*, and *NFATc4* (c2/c3/c4 mutants) using neurofilament (NFM) staining at E10.5 (Figures 1A–1F). Defects were seen in about 70% of c3/c4 mutants and 100% of c2/c3/c4 mutants, but the nature of the defects was similar. No defects in axonal projections were observed at this level of analysis in the single mutants (data not shown). In what follows, we focus on analysis of the triple c2/c3/c4 mutants. The triple-mutant embryos are smaller than stage-matched control littermates, but were at the same Theiler stage, and were not developmentally delayed. The smaller size is likely due to the requirement for calcineurin/NFAT signaling in patterning the vertebrate vasculature (Graef et al., 2001a). Vascular defects often accompany mutations in axonal guidance molecules, apparently reflecting common requirements for patterning the nervous and vascular systems (Behar et al., 1996; von Schack et al., 2001).

At E10.5, most peripheral trigeminal axons observed in the c2/c3/c4 embryos were stunted, but neurite outgrowth appeared to initiate in the correct direction for cranial and dorsal root ganglia (Figures 1A–1F). NFM staining was consistently more intense in the c2/c3/c4 mutant mice, indicating a general increase in NFM production (Figure 1, Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/113/5/657/DC1, and I.A.G. and F.W., unpublished data on NFM mRNA expression).

The central projections of sensory neurons also appeared defective in the mutants. Normally, these axons bifurcate into longitudinal tracts upon reaching the dorsal edge of the hindbrain or the spinal cord and course alongside the gray matter (Figures 1C and 1E). In the c2/c3/c4 triple mutants, in contrast, the central branches of spinal sensory neurons from the DRG failed to project longitudinally upon reaching the dorsal spinal cord at the dorsal root entry zone (DREZ, arrow in Figure 1E). As a result, the longitudinal tract or dorsal funiculus (DF) in wild-type and control littermates was well developed at E10.5 (Figure 1E), but in Theiler stage-matched triple mutants it was absent or very fragmented (Figure 1F and Supplemental Figure S1D available at http://www.cell.com/cgi/content/full/113/5/657/DC1). Similarly, the central projections of trigeminal neurons also appeared defective (Figure 1D, Supplemental Figure S1F available at http://www.cell.com/cgi/content/full/113/5/657/DC1).

The defects in the c2/c3/c4 triple mutants did not appear to be related to a failure of sensory neuron differentiation, because expression of several markers of cell type specification (βIII-*Tubulin*, *Nkx2.2*, *HNF3*β, *Lim1/2, Pax7*, *Islet-1*, *neurogenin-1* and *2*, *Neuro-D*, and *SCG-10*) (Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/113/5/657/DC1) was similar to that observed in littermate controls. Indeed, the neurotrophin receptor genes *TrkB* and *C* were, if anything, overexpressed in c2/c3/c4 mutants (Supplemental Figures S4A–S4B available at http://www.cell.com/cgi/content/full/113/5/657/DC1 and data not shown). The normal expression of most differentiation markers strongly argues against a developmental delay in the NFATc mutant mice.

Triple-mutant embryos also displayed profound disturbances in commissural axon growth as visualized by TAG-1 staining. At E10.5, in control embryos, commissural axons project toward the floor plate and some have already crossed the midline (arrow head and open arrowhead in Figure 1I). In contrast, very few TAG-1 positive neurites can be seen in the mutant and most of them are much shorter as they project only midway in the spinal cord (arrowhead in Figure 1J) and no TAG-1 positive axons reach the floor plate and cross the midline (open arrowhead in Figure 1J). Many NFM positive processes in the mutant were oriented along a mediolateral trajectory (arrow in Figure 1H) and a few axons reached the floor plate. Since these processes are TAG-1 negative, they are unlikely to be misprojections from commissural neurons or motoneurons. Instead they might represent interneurons that have failed to migrate to their proper locations or that are projecting abnormally. Again, neurons in the mutant stain more intensely for NFM.

### Transient Calcineurin Inhibition during Embryonic Development Mimics Sensory Neuronal Defects Seen in *NFATc2/c3/c4* Mutant Mice

Previously characterized functions of the four *NFATc* genes are known to be regulated by the $Ca^{2+}$ activated phosphatase calcineurin, which regulates their nuclear import (Clipstone and Crabtree, 1992; Klee et al., 1998). We therefore examined whether defects seen in triple-mutant mice were due to a failure of transmission of a $Ca^{2+}$/calcineurin signal to the nucleus. We found that *calcineurin B* mutant mice have defects in axonal outgrowth but die at E10.0 due to a failure to properly pattern the developing vascular system (Graef et al., 2001a and data not shown). To circumvent this problem and study the role of calcineurin in axon outgrowth in embryos at later stages, we used the calcineurin inhibi-

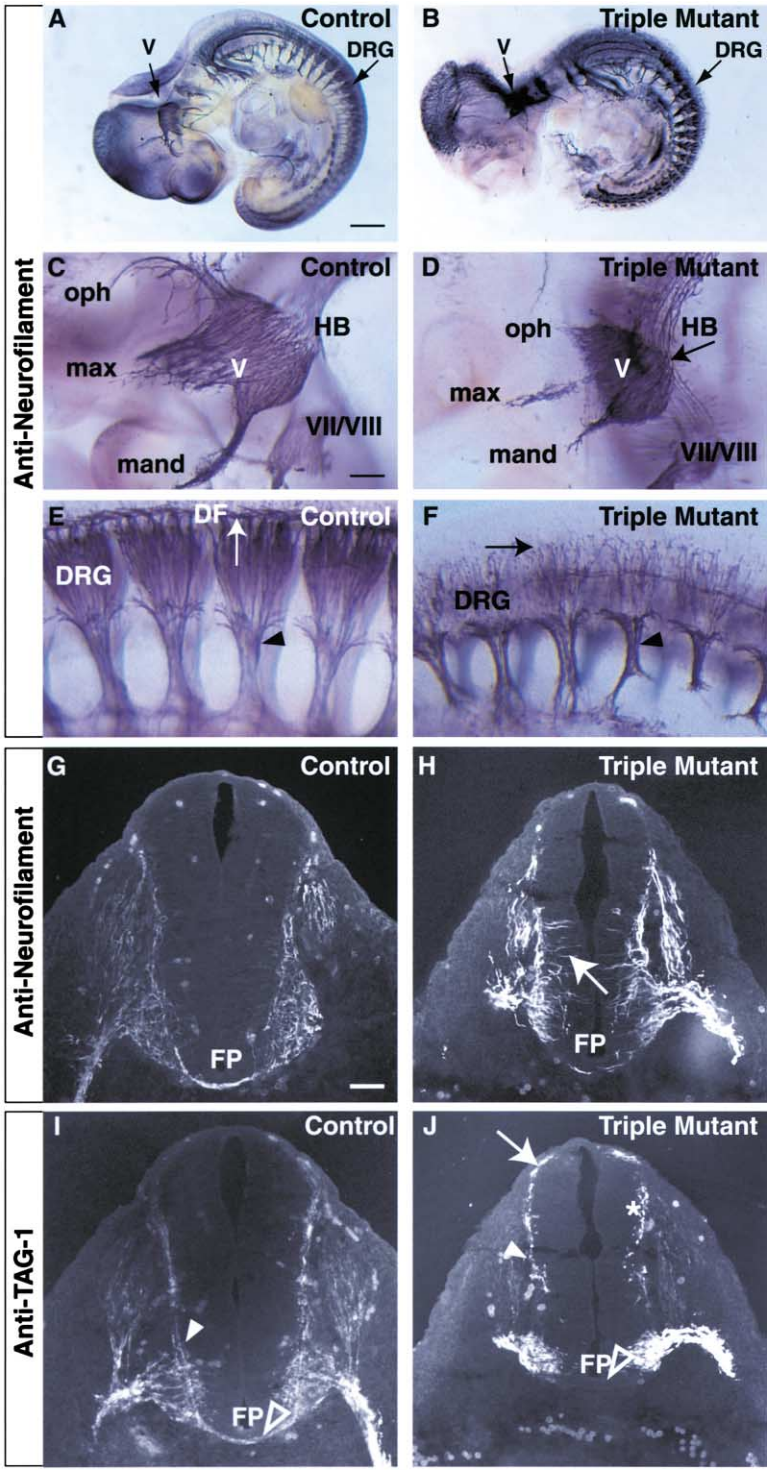



Figure 1. Axon Guidance Defects in NFATc2/3/4 Mutant Embryos

(A–F) Whole-mount immunostaining with anti-neurofilament (NFM) antibody on E10.5 wild-type (A, C, and E) and NFATc mutant (B, D, and F) embryos.

(C–D) The three branches of the trigeminal ganglion (V), (oph, ophthalmic; max, maxillary; and mand, mandibular), fail to extend in NFATc mutant embryos. Arrow in (D) shows descending tracts at the Vth nerve root. HB, hindbrain; rostral is up.

(E–F) Peripheral projections from the dorsal root ganglion (DRG, arrowhead) are severely shortened in the NFATc mutants (F). In mutant embryos (F), sensory afferents from the DRG to the spinal cord (arrow) fail to extend longitudinal tracts alongside the spinal cord. DREZ-dorsal root entry zone (arrow in E) DF-dorsal funiculus dorsal is up.

(G–H) Transverse sections of E10.5 wild-type (G) and mutant (H) embryos stained with anti-NFM antibody. Dorsal is up. Mutant embryos display ventromedially directed projections (arrow in H).

(I–J) Projections of spinal commissural axons (arrowhead in I) to the floor plate (FP), where they cross the midline (open arrowhead), are seen in the control littermates (I). In mutant embryos (J), no commissural axons reach the floor plate. The position of commissural neuron cell bodies is indicated by the asterisk in (J). Some TAG-1 positive processes grow dorsally along the edge of the spinal cord (arrow in J). Scale bar for (A–B) is 1.5 mm; (C–F) is 600 μm; and (G–J) is 50 μm. The difference in intensity of the staining between wt and mutant embryos reflects the higher NFM expression in the mutant embryos and does not represent a difference in exposure.

tor cyclosporin (CsA). CsA is a natural microbial product that crosses the placenta and binds to cyclophilin A, producing inhibitory complexes that block calcineurin phosphatase activity (Liu et al., 1991). Another chemically distinct inhibitor of calcineurin is FK506 (used in later experiments), which binds FKBP12, producing inhibitory complexes (Liu et al., 1991). The exquisite specificity of CsA or FK506 for calcineurin is based on the large and evolutionarily highly perfected composite surface used to bind the calcineurin A/B complex by the CsA/cyclophilin or FK506/FKBP complex (Griffith et al., 1995; Kissinger et al., 1995). Defects observed in E10.5 wild-type embryos treated in utero by administering CsA to the pregnant mothers were indistinguishable from those in *NFATc2/c3/c4* triple-mutant embryos (Figure 2), including a profound impairment of peripheral projec-

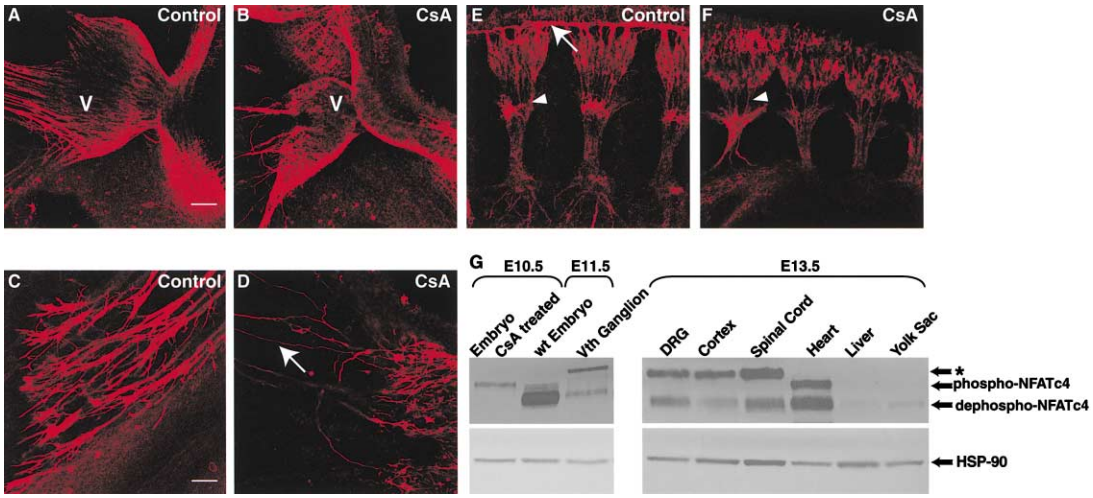



Figure 2. Calcineurin Inhibition during Embryonic Development Phenocopies the NFATc2/c3/c4 Mutant Embryos

(A–F) Whole-mount anti-NFM staining at E10.5 shows sensory axon projection abnormalities in CsA treated embryos.

(A–D) Trigeminal ganglia; rostral is up. Peripheral trigeminal processes are shortened and thin (arrow in D) in the CsA-treated embryos.

(E and F) Dorsal root ganglia; dorsal is up. Peripheral axons from the dorsal root ganglion (arrowhead) fail to extend in CsA treated embryos (F). CsA treated embryos also show a failure of formation of the DF seen in the nontreated age-matched control (arrow in E).

(G) Maternal administration of CsA (lane1) induced phosphorylation of the NFATc4 protein compared to nontreated embryos (lane2). NFATc4 is dephosphorylated in E11.5 trigeminal ganglia (lane 3). The mobility of NFATc4 in E13.5 DRG, cortex, and spinal cord indicates that it is dephosphorylated and active. The E13.5 liver or yolk sac contains little or no NFATc4 while the heart shows a prominent phosphorylated band. The band indicated by the asterisk (*) is a crossreacting band.

Scale bar for (A and B) and (E and F) is 100 μm and for (C–D) is 50 μm.

tions of trigeminal sensory neurons (Figures 2A–2D) and spinal sensory neurons in the DRGs (Figures 2E and 2F). In addition, defects of the central branches of DRG neurons and in specific motor neurons were seen in CsA-treated and c2/c3/c4 triple mutants (data not shown). These observations, together with earlier work (Graef et al., 2001a) indicate that at early stages of embryogenesis calcineurin may be largely dedicated to regulating NFATc function.

If calcineurin were regulating NFATc proteins in growing sensory neurons, we would expect NFATc proteins to be expressed and dephosphorylated in embryonic sensory ganglia. Indeed, NFATc4 was present and dephosphorylated in the trigeminal ganglia, DRGs, cortex, and spinal cord of E13.5 embryos, but was almost undetectable in the liver and other tissues (Figure 2G). In the heart, NFATc4 was partially dephosphorylated (Figure 2G), consistent with the critical role of NFAT signaling in the development of the cardiovascular system (de la Pompa et al., 1998; Graef et al., 2001a). CsA does have access to the developing embryo since treatment of mothers (E7.5–E8.5) results in complete conversion of NFATc4 to the fully phosphorylated form in the embryo (Figure 2G).

### NFATc Is Required Specifically for Neurotrophin-Dependent, but Not for Neurotrophin-Independent Neurite Outgrowth

The defects seen in the c2/c3/c4 mutant mice and the CsA-treated mice could be due either to a defect in production of cues for axon extension by pathway or target cells, or to an impairment of the axons' ability to respond to such stimuli, or both. To test for a cell-autonomous defect, we examined whether the in vivo defects in axon outgrowth were also observed in vitro when the neurons were isolated from their normal environment. We focused on trigeminal ganglia because they are among the first sensory ganglia to form, and are well developed at E10.5 when the triple-mutant embryos are still alive.

Normally, axons from E10.5 trigeminal ganglia are stimulated to extend into a collagen matrix by NGF and NT3, creating a broad axon halo after 48 hr (Figure 3A). In contrast, little outgrowth in collagen was observed from trigeminal ganglia from c2/c3/c4 triple mutants (12.3% ± 1.3% of control explant length), or when wild-type ganglia were cultured in collagen with CsA and FK506 (13.5% ± 1.2% of control explant length) (Figures 3B–3C). To further test for a nonneuronal contribution to the in vitro outgrowth defect (Figures 3B and 3C), we cultured dissociated trigeminal neurons at low density on a two-dimensional laminin substrate. When dissociated trigeminal neurons from c2/c3/c4 mutant E10.5 embryos were cultured on laminin in the presence of NGF and NT3, shorter (9.9% ± 4.2% of control axon length), and fewer axons extended compared to littermate controls (Figures 3E and 3H). Calcineurin was also necessary for outgrowth under these culture conditions, because treatment of wild-type trigeminal neurons with FK506 and CsA (10.2% ± 4.7% of control axon length) mimicked the outgrowth defects seen in the c2/c3/c4 mutant neurons (Figures 3F and 3I). Together, these experiments show that loss of NFATc2/c3/c4 gene function, or inhibition of calcineurin, can impair axon outgrowth when neurons are cultured in vitro. This is observed even in low-density cultures where it is difficult to argue for effects via nonneuronal cells, strongly implying that at least some of the defects observed in vivo are cell-autonomous.

Pharmacological inhibition of axon outgrowth in ex-

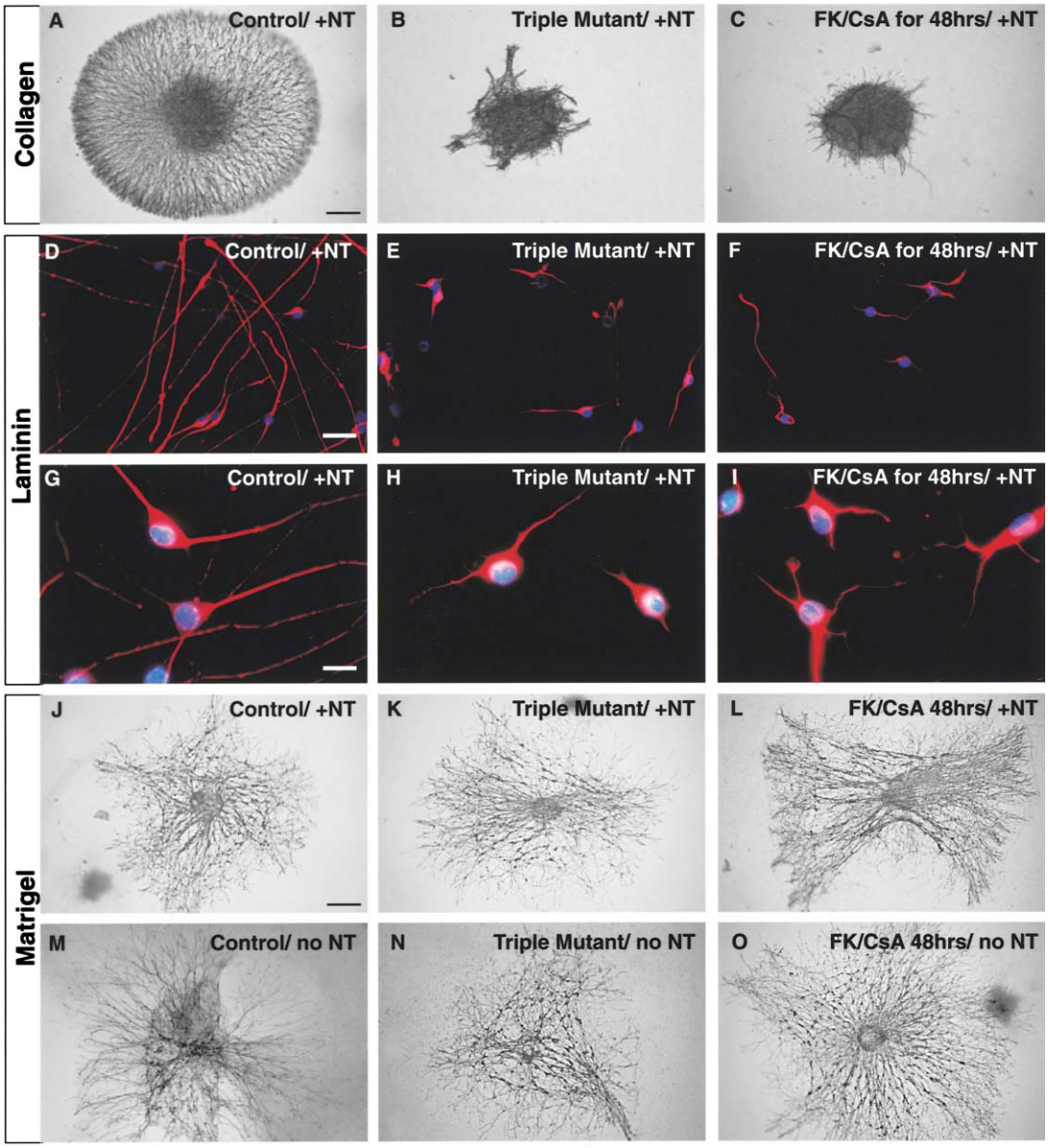



Figure 3. Cell Autonomous Defect of Sensory Axon Growth

(A–C) E10.5 trigeminal explants were grown for 48 hr in collagen. Axon outgrowth was visualized by staining with anti-NF-M antibody. (A) Wild-type ganglia; (B) NFATc triple-mutant ganglia; (C) Wild-type explants treated with FK/CsA at the onset of the culture period.

(D–I) Dissociated trigeminal neurons cultured at low density on laminin and stained with anti-NF-M antibody (red) and DAPI (blue). (D and G) littermate control; (E and H) NFATc mutant cells; (F and I) cells treated for 24 hr with FK/CsA.

(J–O) Trigeminal explants cultured in matrigel. Neither mutation of NFATc2/c3/c4 (K) nor treatment of explants with FK/CsA (L) in the presence of NT-3 and NGF affected axon elongation in matrigel. Explants cultured in the absence of NT-3 and NGF also showed no impairment of neurite growth in matrigel (M–O).

Scale bar for (A) is 500 μm; (B–C) 250 μm; (D–F) 30 μm; (G–I) 10 μm; and (J–O) 650 μm.

plant and dissociated cultures described above required use of both CsA and FK506 and only partial block was observed with either alone (data not shown). This contrasts with the ability of CsA by itself to inhibit sensory axon growth over longer periods in vivo (Figure 2). This difference might be explained by the observation that long-term blockage of NFAT signaling suppresses expression of calcineurin and NFATc4 in our studies (Figure 2G, G.R.C. and I.A.G., unpublished data and Zeng et al., 2001), while short-term treatment does not. Hence, complete inhibition of calcineurin in neurons at E10.5 in vitro might require both drugs to form enough inhibitory complexes to neutralize calcineurin, while prolonged treatment in vivo requires only CsA treatment.

Axon outgrowth in the in vitro assays just described and outgrowth of the peripheral branches of sensory axons in vivo are dependent on neurotrophins (Kaplan and Miller, 2000; O'Connor and Tessier-Lavigne, 1999). This raised the question whether calcineurin/NFAT signaling is required for outgrowth stimulated by neurotrophins. We took advantage of our previous observation that embryonic sensory axons will extend profusely

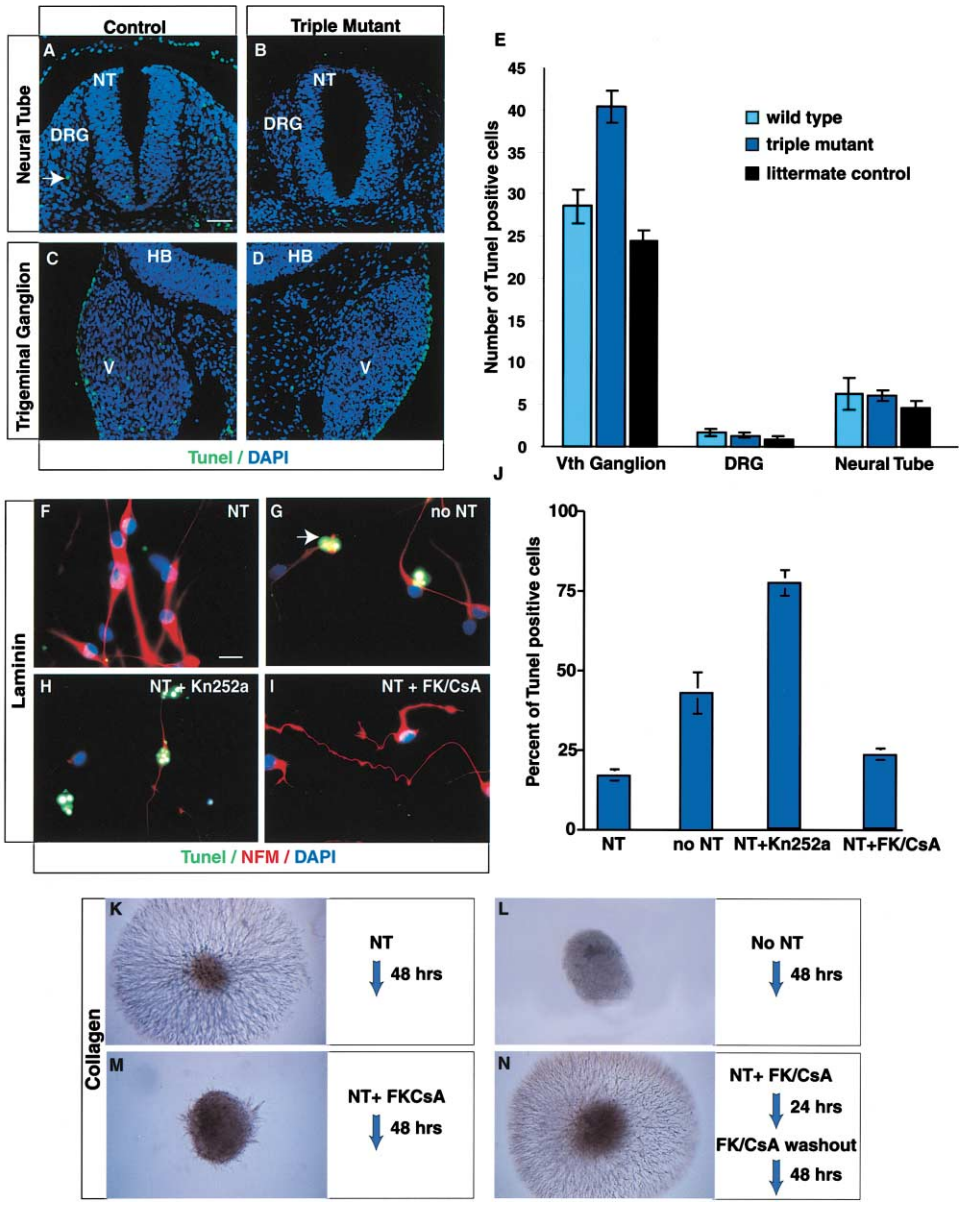



Figure 4. Neither Calcineurin nor NFATc2/c3/c4 Are Required for Sensory Neuron Survival In Vivo or In Vitro

(A–D) Tunel (green) and nuclear (DAPI, blue) stain of E10.5 transverse sections; dorsal is up. Tunel-positive cells are indicated by arrow in (A), (NT)-neural tube.

(E) Bars represent the mean number of Tunel positive cells per section for the indicated structures (DRG, n = 12; NT, n = 6; and Vth Ganglion, n = 5) +/− SEM.

(F–I) Survival of E10.5 dissociated trigeminal neurons cultured under neurotrophin-dependent conditions for 24 hr. Cell death was assessed by Tunel-staining (green), anti-NF-M staining (red), and nuclear stain (DAPI, blue).

(J) Quantitation of the number of Tunel-positive cells in the cultures shown in (F–I). The mean ± SEM of triplicate cultures, scored blindly, is shown.

(K–N) Wild-type E10.5 trigeminal explants were grown for 48 hr in a three-dimensional collagen matrix. Axonal outgrowth was visualized by anti-NF-M staining; (K), non-treated control; (L), cultures without NT-3 and NGF; (M), FK/CsA + NT throughout the culture period; (N), cultures in which FK/CsA is washed out after a period of 24 hr.

Scale bar for (A) through (D) is 50 μm; for (F) through (I) is 10 μm; for (K and N) is 500 μm; and for (M and L) 200 μm.

in the absence of neurotrophins if they are grown in matrigel (Figures 3J and 3M), a basement membrane extract that contains several extracellular matrix proteins. Strikingly, extension of axons from c2/c3/c4 mutant trigeminal ganglia on this substrate was normal (Figure 3K). Similarly, inhibition of calcineurin did not affect trigeminal axon outgrowth on this substrate (Figure 3L). The difference between outgrowth on matrigel compared to either collagen or laminin is that outgrowth on matrigel did not require neurotrophins (Figures 3M–3O). Further, these results demonstrate that neurons from the NFATc mutant trigeminal ganglia as well as FK/CsA-treated trigeminal neurons are not generally sick or growth-arrested. These results contrast markedly with

the profound impairment seen for the neurotrophin-dependent outgrowth on laminin or in collagen (Figures 3A–3I) and suggest that neurotrophins might produce their effects on axonal outgrowth in part by signaling through calcineurin and NFATc proteins.

### NFAT Signaling Is Not Essential for Neurotrophin-Dependent Survival In Vivo or In Vitro

Since neurotrophins induce neurite outgrowth and promote survival during development, we determined if NFAT signaling was required for the survival effects of neurotrophins. Cell death is a normal part of CNS and PNS development and can be observed by TUNEL staining of sections of control mice at E10.5. In the mutant mice, we did not observe a change in the number of TUNEL positive neurons in the DRGs or neural tube of mutants and only a very slight increase in the trigeminal ganglia (Figures 4A–4E). Therefore, cell death is not the primary reason for the inability of axons to project to the periphery. This result is consistent with the observation that growth of mutant E10.5 trigeminal ganglia in matrigel is similar to that observed in controls (Figures 3K and 3N).

To further define the role of NFAT signaling on survival, we used neurotrophin-dependent low-density cultures of dissociated E10.5 trigeminal neurons in serum-free medium (Buchman and Davies, 1993), in the presence or absence of NGF and NT3. Culturing the cells without neurotrophins more than doubled the amount of cell death in the cultures (Figures 4G and 4J). An even greater degree of cell death was induced by 150 nM Kn252a, an inhibitor of Trk kinase activity (Figures 4H and 4J). Thus, survival in these cultures is highly dependent on neurotrophins. In contrast, the combination of FK/CsA that completely blocks calcineurin activity and axon outgrowth did not increase cell death (Figures 4I and 4J). These data suggest that, while neurotrophin signaling under these culture conditions is essential for neuronal survival, calcineurin signaling is dispensable.

If calcineurin were not required for survival one would predict that the effects of FK/CsA inhibition of calcineurin should be fully reversible. To test this prediction, trigeminal ganglia cultured on collagen were treated with FK/CsA for 24 hr and then the drug removed by washout. Treatment of trigeminal ganglia for 48 hr with FK/CsA completely blocked axonal outgrowth (Figure 4M). However, when the drugs were washed out after 24 hr, axonal outgrowth recovered fully after a further 48 hr (compare Figures 4M and 4N). In contrast, trigeminal ganglia cultured for 48 hr in the absence of neurotrophins showed near complete lack of outgrowth (Figure 4L). Thus, FK/CsA treatment and block of calcineurin/NFAT signaling for 24 hr does not lead to significant cell death or irreversible toxicity. These results indicate that calcineurin/NFAT signaling is selectively required for neurotrophin-dependent axon outgrowth but not neurotrophin-dependent survival.

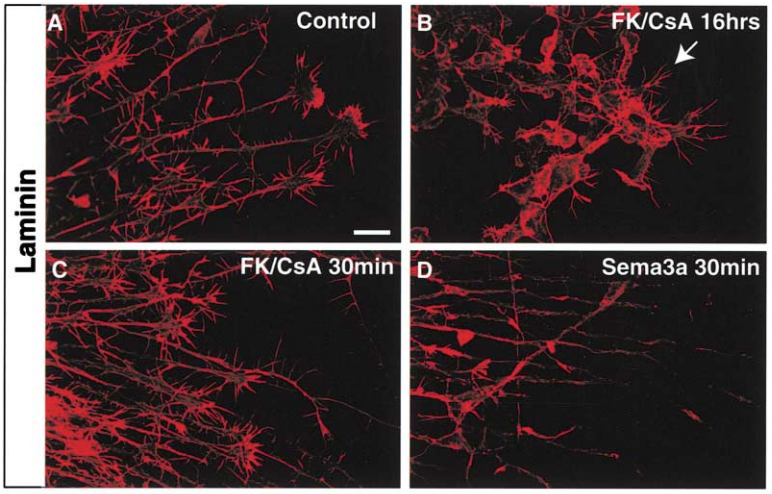
### Delayed but Specific Effects of Calcineurin Inhibition

A loss of NFATc function most likely results in impaired axon outgrowth because of failure to transcribe genes essential for neurite outgrowth and axon extension. However, pharmacological inhibition of calcineurin with FK/CsA could potentially impair axon outgrowth either by affecting NFAT-dependent transcription, or through a direct effect on the axons or growth cones. To test for a direct effect on axons, we first examined whether the failure of axonal outgrowth on laminin with FK/CsA reflects growth cone collapse or retraction. When cultures of trigeminal neurons were treated with FK/CsA for 16 hr, only a few growth cones formed and elongation was absent (Figure 5B). However, addition of FK/CsA for 30 min after 16 hr in culture, did not induce collapse of extending growth cones (Figure 5C), in contrast to treatment with Sema3A (Figure 5D). Thus, FK/CsA did not produce an immediate collapsing effect on the cytoskeleton. The fact that NFAT/calcineurin signaling was not required for axon outgrowth on matrigel allowed us to test for a role of this signaling pathway in acute semaphorin responses. Sema3A also induced efficient collapse of growth cones in cultures of trigeminal ganglia from c2/c3/c4 triple mutants (data not shown) or of wild-type trigeminal ganglia cultured with CsA and FK506 (Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/113/5/657/DC1) on matrigel, demonstrating that only selective signal transduction pathways are affected by lack of calcineurin/NFATc signaling.

Additional evidence for a transcriptional role of calcineurin came from experiments in which we determined the lag time between addition of FK/CsA and the arrest of axon outgrowth. We grew wild-type trigeminal ganglia for 24 hr in collagen gels, then added FK/CsA and followed the further growth ($\Delta$) of the axons at the indicated times (t + 24 hr) (Figure 5E). Quantitative analysis showed that after drug addition axonal elongation proceeded for five hours at the same rate as that observed with the nontreated explants, but at that point outgrowth slowed by a factor of 3.5 in the drug-treated explants (Figure 5E). The delay in the onset of action of FK/CsA, and the lack of acute collapse-inducing activity, argue against a direct effect of calcineurin inhibition on axon elongation and is consistent with a model in which the inhibitory effects of calcineurin on neurite outgrowth are transcriptional.

### NFATc Functions Downstream of Neurotrophins

The finding that calcineurin/NFAT signaling was required for the neurotrophin-induced outgrowth but not the neurotrophin-independent outgrowth of E10.5 trigeminal axons led us to examine whether NFAT signaling is activated by neurotrophins. We tested this possibility using cultured E15.5 cortical neurons, because, unlike sensory neurons, they are not dependent on neurotrophins for their survival in culture, yet they express TrkB receptors on their surface. We found that BDNF treatment induces nuclear translocation of EGFP-tagged NFATc4 within 30 min as reflected by the disappearance of the clear nucleus in BDNF-treated cells (Figure 6A, top middle panel). NGF did not induce translocation, consistent with lack of expression of TrkA (data not shown). However, when EGFP-NFATc4 (green) and TrkA (red, bottom panel) were introduced into the cells by cotransfection, NGF led to rapid translocation of EGFP-NFATc4 into the nucleus (Figure 6A, middle panel). Addition of CsA and FK506 to the cultures blocked translocation (Figure 6A, right panel). These



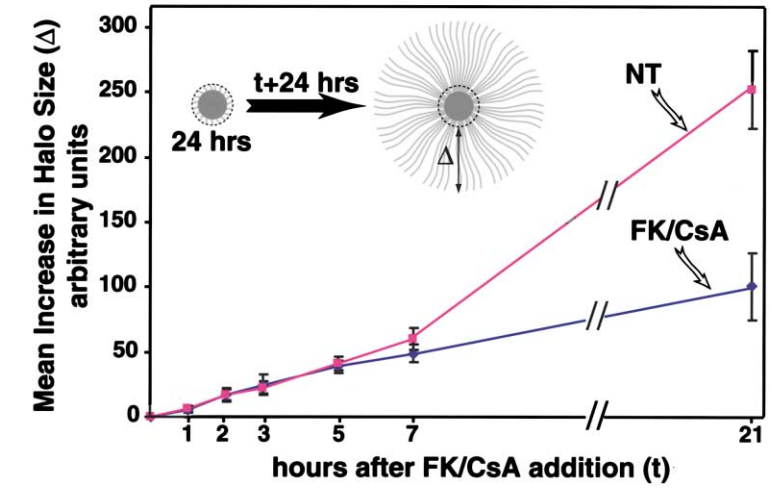




Figure 5. Inhibition of Neurite Outgrowth by CsA Requires Several Hours

Cubes of wild-type trigeminal explants cultured on laminin, and growth cones were visualized by staining for F-actin with phalloidin (red).

(A) control culture; (B) addition of FK/CsA at the onset of culturing (arrow indicates a growth cone next to the cell body); (C) short-term treatment (30 min) with FK/CsA; (D) Sema-3A-30 min. Scale bar for (A–D) is 20 μm.

(E) Graph shows the mean change in neurite length (Δ) relative to the 24 hr time point ± S/D of FK/CsA-treated and nontreated explants as a function of time (t) after drug addition (means were generated from three independent experiments, n = 9 for FK/CsA and n = 6 for nontreated explants). Neurite length was calculated by subtraction of neurite length measured after 24 hr of growth (before drug addition, time point 0) from neurite length at the indicated time points after drug addition.

results demonstrate that neurotrophins act directly in neurons to induce calcineurin activity and regulate NFATc4 nuclear localization.

Translocation of NFATc proteins to the nucleus is one of two stimuli that are required for activation of NFAT transcription complexes. The second stimulus usually requires ras or protein kinase C (PKC) activation (Crabtree, 1989). Since neurotrophins can activate ras and PKC, it seemed possible that they might provide the two stimuli necessary for NFAT-dependent transcription. We found that BDNF was a powerful activator of NFAT-dependent transcription in E15.5 cultured cortical neurons (Figure 6B). BDNF-induced, NFAT-dependent transcription was blocked by FK/CsA (Figure 6B) at concentrations that did not inhibit the expression of a constitutively active luciferase reporter gene (data not shown). NGF did not activate transcription from this reporter unless TrkA was introduced into the cells by transfection (Figure 6C). Thus, neurotrophins can stimulate NFATc nuclear translocation and activation of NFAT-dependent transcription in cortical neurons, demonstrating a direct action of neurotrophins on NFAT-dependent transcription.

TrkA receptors transfected into cortical neurons, which lack endogenous TrkA receptors, required the PLCγ1 interaction site (Y794) or the Shc-interaction site (Y499) to activate NFAT-dependent transcription (Figure 6C) in response to NGF. The requirement for the PLCγ1 interaction site may relate to PLCγ's ability to stimulate $Ca^{2+}$ release and the fact that $Ca^{2+}$ is essential to activate calcineurin and induce translocation of the cytosolic subunits of NFATc transcription complexes. A requirement for the Shc-interaction site might reflect the requirement for ras /MAPK or PI3K activation for inducing the nuclear components of NFATc transcription complexes, which are PKC/ras-dependent. As a control, activation of an AP-1 reporter, which is dependent on Ras-signaling, was not affected by mutation of the PLCγ1-interaction site on TrkA, but was blocked by the Shc-interaction site mutation (data not shown).

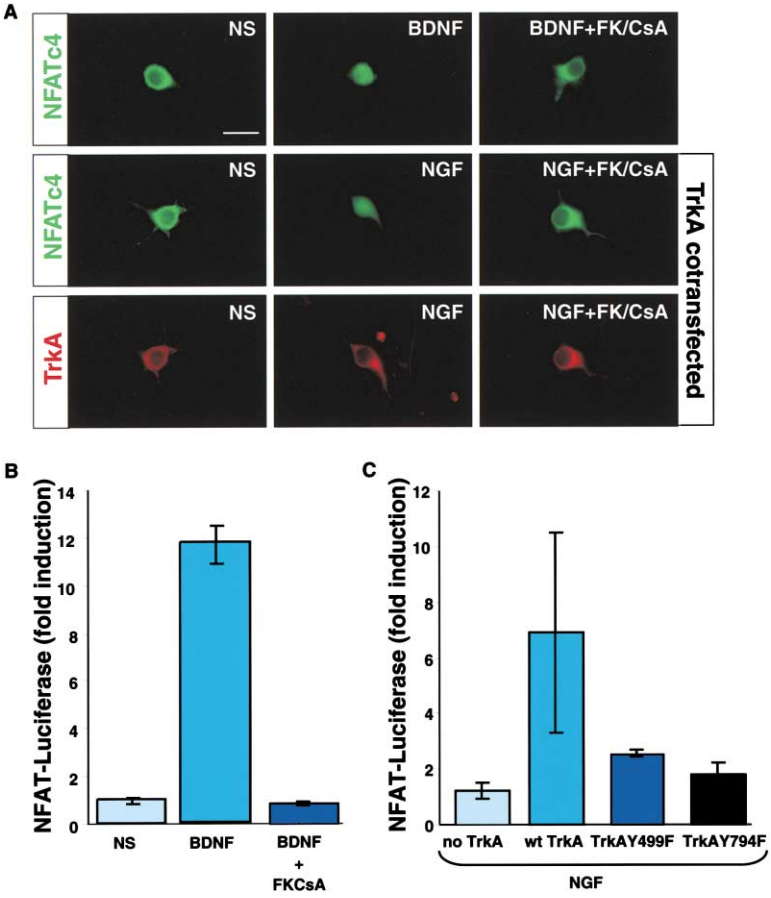



Figure 6. Neurotrophins Regulate NFATc Translocation and Transcriptional Activation

(A) Neurotrophin-induced cytoplasmic-to-nuclear translocation of EGFP-NF-ATc4 in cortical neurons. Top row: representative images showing NF-ATc4 distribution (green) before stimulation ("NS", left), following 30 min of stimulation with 100 ng/ml BDNF ("BDNF", middle) and in the presence of FK/CsA ("BDNF+FK/CsA", right). Middle row: Nuclear translocation of NFATc4 (green) upon stimulation with 100 ng/ml NGF in cortical neurons that were cotransfected with wild-type TrkA. The addition of FK/CsA inhibited the nuclear translocation of NFATc4 induced by NGF stimulation (right). Bottom row: co-transfected wild-type TrkA construct (red). Scale bar for (A) is 10 μm.

(B) Activation of NFAT-dependent transcription by BDNF (18 hr) in cortical neurons assessed using a transfected NFAT-luciferase reporter plasmid.

(C) Activity of TrkA mutants on NFAT-dependent transcription in cortical neurons treated for 18 hr with NGF.

### Calcineurin Inhibition also Impairs Netrin-Dependent Axon Outgrowth

The defects seen in the NFATc null mice and in the CsA-treated embryos were more extensive than those expected if calcineurin and NFATc were only required for neurotrophin signaling. For example, extension defects of commissural axons in c2/c3/c4 null mice are similar to defects found in mice mutant in netrin-1 or its receptor, DCC (Fazeli et al., 1997; Serafini et al., 1996) (Figures 1H and 1J). We found that the calcineurin inhibitors FK/CsA blocked the rapid (19 hr) netrin-induced axon extension from E13 rat dorsal spinal cord explants in collagen and matrigel three-dimensional cultures (Figures 7B and 7D). However, netrin-independent outgrowth (Keino-Masu et al., 1996), which is very slow and can be measured at 43 hr, was not blocked by FK/CsA (Figures 7A and 7C). These data indicate that the observed inhibitory effect does not represent a general inhibition of outgrowth, but rather inhibition of outgrowth stimulated by netrin/DCC signaling (also see Discussion).

We found that netrin activated endogenous NFAT-dependent transcription by about 2- to 3-fold in E15.5 cortical neurons (Figure 7E). This increase appeared to be calcineurin-dependent since it was blocked by FK/CsA. Because cortical neurons may not have saturating levels of the netrin receptor DCC, we cotransfected DCC with the reporter construct and found that netrin induced about a 4- to 5-fold increase in NFATc activity (Figure 7E), suggesting that DCC was limiting in E15.5 cortical neurons. To further determine if transcription was dependent on netrin, we cotransfected a dominant-negative version of DCC lacking its cytoplasmic domain (Dn DCC) and found that it blocked NFAT-dependent transcription (Figure 7E). These observations indicate that netrin is a powerful activator of endogenous NFAT-dependent transcription in cultured cortical neurons.

Together, these observations suggest that the defects in commissural axon outgrowth observed in vivo in NFATc triple-mutant embryos could be due partly or even entirely to loss of NFATc function in neurons. We cannot yet fully exclude that the failure of commissural axon growth could in part reflect a defect in presentation of cues in the environment; however, we have found that expression of netrin-1 and DCC mRNAs were normal in E10.5 triple mutants (Supplemental Figures S4C–S4F available at http://www.cell.com/cgi/content/full/113/5/657/DC1).

## Discussion

We have presented several lines of evidence that signaling through calcineurin and NFATc proteins play critical roles in regulating embryonic axon outgrowth from a variety of neuronal classes. Based on these data, we

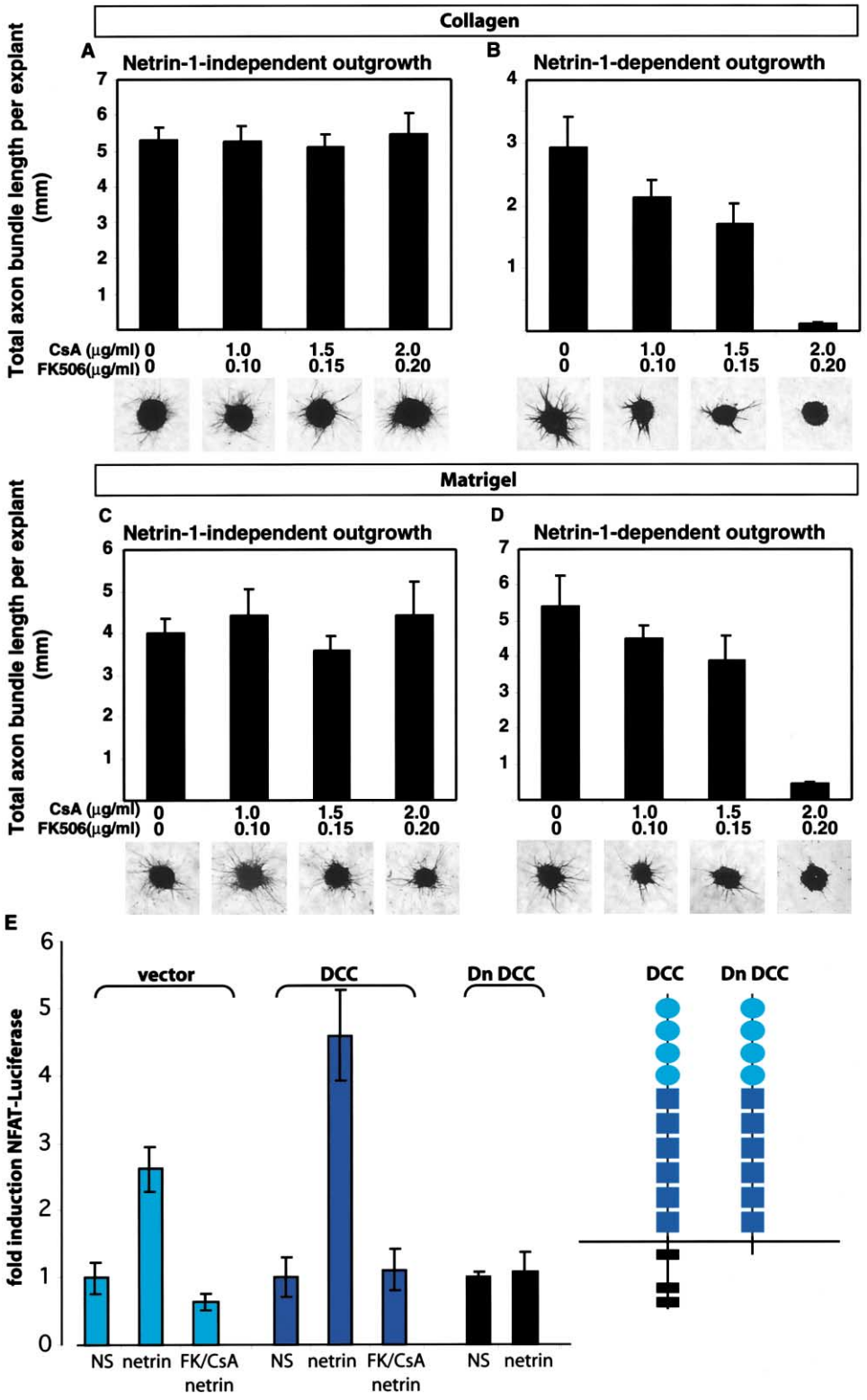



Figure 7. Inhibition of Calcineurin Specifically Blocks Netrin-dependent but not Netrin-Independent Growth from Dorsal Spinal Cord Explants

(A and C) Calcineurin inhibition has no effect on netrin-independent axon outgrowth from dorsal spinal cord explants in either collagen or matrigel. E13 rat spinal cord explants were cultured for 43 hr in collagen (A) or matrigel (C) with increasing concentrations of FK/CsA.

(B and D) FK/CsA treatment blocks netrin-dependent commissural axon outgrowth in a dose-dependent manner. E13 rat spinal cord explants were cultured for 19 hr in collagen (B) or matrigel (D) in presence of netrin-1 (100 ng/ml) and increasing concentrations of FK/CsA. The total axon bundle length per explant was measured from at least 10 explants obtained from two independent experiments. Images of representative anti-NF-M stained explants are shown below the relevant bar for each condition.

(E) Netrin activates NFAT-dependent transcription in E15.5 cortical neurons in a calcineurin and DCC-dependent manner. Stimulation with 200 ng/ml recombinant netrin-1 activates NFAT-dependent transcription, this transcriptional induction is blocked by either FK/CsA treatment at the time of stimulation or cotransfection of Dn DCC. Cartoon depicts wild-type DCC and Dn DCC, which lacks the cytoplasmic domain of wild-type DCC.

propose that embryonic axon outgrowth stimulated by growth factors such as neurotrophins and netrins requires these factors not only to stimulate the tips of growth cones, but also to selectively activate a calcineurin/NFAT-dependent transcriptional program controlling the rate of axonal extension (Figure 8).

### NFAT Signaling Functions in Neurons to Promote Embryonic Axon Growth

The dramatic defects in nervous system development in NFATc2/c3/c4 triple mutants and in CsA-treated embryos (Figures 1 and 2), do not appear to result from defects in cell specification, as assessed by the normal expression of a variety of markers of neuronal identity (Supplemental Figure S2 available at http://www.cell.com/cgi/content/full/113/5/657/DC1). The defects also do not reflect a major increase in cell death, because we did not observe enhanced apoptosis in mutant embryos. Furthermore, inhibition of calcineurin/NFAT signaling with FK/CsA reversibly blocks sensory axon growth from explants in collagen and does not increase sensory neuron death in low-density cultures (Figure 4). The idea that the primary defect is a defect in axon growth is further supported by the appearance of mutant sensory ganglia, which have an apparently normal shape despite the short length of axons (Figure 1).

In principle, the growth defects in vivo in NFATc triple mutants could result from a defect in the neurons, a defect in the environment through which their axons must grow, or both. Our in vitro data strongly suggest that the in vivo phenotypes reflect at least partly a defect in the neurons, since outgrowth into collagen of trigeminal or commissural axons from explants in response to neurotrophins or netrins is inhibited by pharmacological (and in the case of trigeminal ganglia genetic) blockade of NFAT signaling (Figures 3 and 7). These in vitro cultures are believed to be representative models of growth of these axons in their normal environments. These results are consistent with calcineurin/NFAT signaling being required in the neurons themselves, although they do not establish this point conclusively, since those explants contain nonneuronal cells as well. More conclusive evidence for a cell-autonomous requirement for NFAT signaling in axon growth is, however, provided in the case of sensory axons by axon outgrowth defects in low-density cultures (Figure 3), where invoking indirect effects via nonneuronal cells is even less plausible. While these results thus support a cell-autonomous requirement for calcineurin/NFAT signaling in outgrowth of these axons in vivo, we currently cannot exclude an additional role for NFAT signaling in surrounding or supporting cells. In preliminary studies, however, we have not found a change in expression of neurotrophins, netrins, or their receptors in the triple mutants. Further studies will be required to determine whether environmental defects contribute in any way to the axon outgrowth defects seen in vivo.

### How Does NFATc Participate in Cell-Autonomous Regulation of Axon Growth?

At one extreme, NFATc proteins could perform a general function in neurons that simply affects axon growth in some indirect manner. However, the following lines of evidence indicate a more central role for NFATc proteins in the sustained transduction of signals for axon growth downstream of growth factors like neurotrophins and netrins.

First, the phenotype of NFATc triple-mutant mice can be mimicked by in vivo inhibition of calcineurin through administration of CsA to embryos, indicating that calcineurin-regulated NFATc activity is specifically important for the in vivo phenotype. The same is true in vitro, where outgrowth defects of triple-mutant trigeminal ganglia are reproduced by pharmacological calcineurin inhibition of wild-type ganglia. The reversibility of the pharmacological inhibition, and the ability to shut down outgrowth with late addition of FK/CsA, demonstrate that ongoing stimulation of calcineurin/NFAT signaling is required for axon outgrowth in vitro and in vivo.

Second, neurotrophins and netrins directly activate NFAT transcriptional activity in vitro, presumably by inducing NFATc4 nuclear translocation (as demonstrated directly for neurotrophins but not yet netrins). Intracellular $Ca^{2+}$ transients elicited by neurotrophins and netrins have an important role in regulating growth cone motility and axon growth (Hong et al., 2000; Lankford and Letourneau, 1989; Ming et al., 2002), and might also underlie the activation of calcineurin/NFAT-dependent transcription by these factors.

Third, NFAT signaling is required for growth factor-dependent, but not growth factor-independent extension of sensory and commissural axons. This idea is most strongly supported in the case of trigeminal sensory neurons, since genetic and pharmacological blockade of calcineurin/NFAT signaling both impair neurotrophin-dependent growth of these axons in collagen or on laminin, but not their neurotrophin-independent growth in matrigel. Clear, though less complete, evidence for this idea was also obtained in the case of commissural neurons, since NFAT-signaling is required for the netrin-dependent but not the slow netrin-independent extension of commissural axons in collagen.

Taken together, these results support a model in which neurotrophins and netrins, in addition to their direct actions on growth cone tips, must activate a calcineurin-NFAT-dependent transcriptional program that is required in an ongoing way for efficient embryonic axon outgrowth in response to these factors (Figure 8).

The impairment of trigeminal axon growth in vivo in NFATc triple mutants is greater than might be expected from complete loss of neurotrophin signaling (compare Figure 1 with O'Connor and Tessier-Lavigne, 1999; Tucker et al., 2001), and the impairment of commissural axon growth in these animals is also more severe than in either *netrin-1* or *DCC* mutant embryos (compare Figure 1 and Fazeli et al., 1997; Serafini et al., 1996). These observations suggest that other growth factors (X in Figure 8) collaborate with neurotrophins and netrins to stimulate the extension of trigeminal and commissural axon growth in vivo, and that these factors also must activate NFAT signaling to produce their effects. These considerations raise the possibility that activation of calcineurin/NFAT signaling might be required quite generally for stimulation of embryonic axon outgrowth by growth factors.

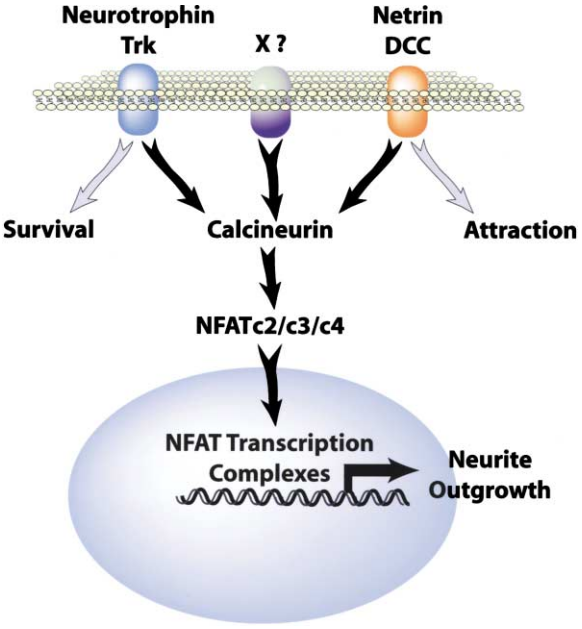



Figure 8. $Ca^{2+}$, Calcineurin and NFATc Transduce Signals for Neurite Outgrowth

Model of signaling by netrins and neurotrophins. Calcineurin and NFATc are essential for netrin- and neurotrophin-dependent neurite outgrowth but appear to have little or no role in neurotrophin-induced survival or rapid growth cone attraction or repulsion. Based on the observation that the c2/c3/c4 mutant phenotypes were more severe than either neurotrophin or netrin mutants we propose that other factors (X?) also require calcineurin NFAT signaling to induce outgrowth.

### Independent Control of Axonal Extension and Survival: a Rationale for the Selectivity of NFAT Signaling

Although sensory neurons lacking NFAT signaling are unable to extend axons efficiently in response to neurotrophins, they do not appear to be compromised in their ability to interpret the survival promoting activity of neurotrophins. Indeed, in vivo, the dramatic defects in axon extension seen in *NFATc2/c3/c4* mutants are not accompanied by a dramatic increase in cell death. This precise parsing of signals for survival and for axon extension could allow independent control of these two processes by factors encountered along the paths of axons to their targets and independent regulation of these two effects for a given factor. Independent control of these two processes is in fact observed. For example, embryonic sensory axons initially respond to neurotrophins with rapid axon outgrowth, but when they reach their targets they stop extending rapidly in response to these factors (instead responding by elaborating their terminal arbors) at the same time as they actually become more dependent on neurotrophins for their survival. A switching off of the calcineurin/NFAT signaling pathway could in principle underlie the switch from an elongating to an arborizing mode in these neurons, without affecting their trophic dependence on neurotrophins.

Our data thus define a dedicated signaling and transcriptional program required for growth-factor-stimulated axon outgrowth of embryonic axons. The finding of such a program was surprising, as we believe it has been implicitly assumed that the ability of embryonic neurons to extend an axon in response to growth-stimulating factors is simply another generic aspect of an intrinsic neuronal specification program. This implicit assumption was perhaps reinforced by the evidence that growth factors like neurotrophins stimulate axon extension by acting on growth cones at the tips of axons, far from their cell bodies (e.g., Campenot, 1977). Our results indicate that, while likely necessary, stimulation of axon tips is not apparently sufficient for sustaining the rapid growth induced by the growth factors, and that sustained activation of NFAT-dependent transcription is also required.

#### Experimental Procedures

See Supplemental Data available at http://www.cell.com/cgi/content/full/113/5/657/DC1.

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