Neuron
Article

Isoform-Specific Dephosphorylation of Dynamin1 by Calcineurin Couples Neurotrophin Receptor Endocytosis to Axonal Growth

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DOI 10.1016/j.neuron.2011.04.025

SUMMARY

Endocytic events are critical for neuronal survival in response to target-derived neurotrophic cues, but whether local axon growth is mediated by endocytosis-dependent signaling mechanisms remains unclear. Here, we report that Nerve Growth Factor (NGF) promotes endocytosis of its TrkA receptors and axon growth by calcineurin-mediated dephosphorylation of the endocytic GTPase dynamin1. Conditional deletion of *calcineurin* in sympathetic neurons disrupts NGF-dependent innervation of peripheral target tissues. Calcineurin signaling is required locally in sympathetic axons to support NGF-mediated growth in a manner independent of transcription. We show that calcineurin associates with dynamin1 via a PxIxIT interaction motif found only in specific dynamin1 splice variants. PxIxIT-containing dynamin1 isoforms colocalize with surface TrkA receptors, and their phosphoregulation is selectively required for NGF-dependent TrkA internalization and axon growth in sympathetic neurons. Thus, NGF-dependent phosphoregulation of dynamin1 is a critical event coordinating neurotrophin receptor endocytosis and axonal growth.

INTRODUCTION

Neurotrophins are trophic factors secreted by target tissues that coordinate multiple aspects of neuronal development, including cell survival, axonal and dendritic growth, and synapse formation (Huang and Reichardt, 2001). In polarized neurons, neurotrophins elicit their effects by activating signaling pathways characterized by their subcellular site of action (Heerssen and Segal, 2002). Local signaling in distal axons and growth cones mediates acute responses including rapid axon growth, branching, and guidance. In contrast, retrograde signaling to the cell body and nucleus elicits long-term changes in gene expression necessary for neuronal survival and differentiation. The neurotrophin, NGF, secreted by peripheral target tissues, supports survival of sympathetic and sensory neurons by regulating endocytosis and retrograde vesicular trafficking of NGF:TrkA complexes (Zweifel et al., 2005). Although much is known about the mechanisms regulating retrograde survival signaling to the nucleus, how target-derived NGF activates TrkA receptors in nerve terminals to induce axonal outgrowth remains unclear.

In the developing sympathetic nervous system, the neurotrophins NT-3 and NGF act through the same TrkA receptor to orchestrate sequential stages of axon growth (Glebova and Ginty, 2005; Kuruvilla et al., 2004). NT-3, which is highly expressed in intermediate targets such as the vasculature, promotes early stages of axon growth. NGF, which is highly expressed in final peripheral targets, supports final target innervation (Glebova and Ginty, 2004; Kuruvilla et al., 2004). Unlike NGF, NT-3 cannot promote endocytosis and retrograde transport of TrkA (Kuruvilla et al., 2004). Although both NGF and NT-3 promote robust axon growth in sympathetic neurons, only NGF supports neuronal survival. Thus, differential trafficking of TrkA seems to be responsible only for differences in the ability of NGF and NT-3 to promote neuronal survival. Consistent with the idea that activation of cell-surface TrkA receptors is sufficient to support local axonal growth, NGF immobilized on beads elicits acute axonal responses, including growth cone extension, branching, and guidance (Gallo et al., 1997; Gallo and Letourneau, 1998). However, axon growth along intermediate targets is characteristically distinct from final stages of target innervation (Rubin, 1985). Furthermore, NGF- and NT-3-treated neurons display distinct morphological responses (Orike et al., 2001). Currently, it remains unclear whether NGF and NT-3 employ distinct signaling mechanisms downstream of a common TrkA receptor to promote axonal growth. In particular, the contribution of endocytic trafficking of TrkA receptors to neurotrophin-mediated axonal growth remains poorly defined.

In sensory neurons, a calcineurin/NFAT-dependent transcriptional program has been reported to control axonal growth in response to NGF and NT-3 (Graef et al., 2003). Calcineurin is a calcium-responsive serine/threonine phosphatase, consisting of a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurinB). Ca^{2+}-dependent activation of calcineurin results in dephosphorylation and nuclear import of NFAT transcription factors (NFAT1-4) (Flanagan et al., 1991). Mice deficient in calcineurin/NFAT signaling show defects in neurotrophin-dependent sensory axon growth, without any disruption of neuronal differentiation or survival (Graef et al., 2003). Although NFAT has received the most attention among calcineurin substrates,

calcineurin has many other downstream targets that may play important roles in neuronal development (Li et al., 2011). Here, we identify a new endocytic mechanism by which calcineurin regulates neurotrophin-dependent axonal growth. We found that calcineurin activity is specifically required for NGF-mediated, but not NT-3-mediated, axon growth in sympathetic neurons. We identified dynamin1 as a local target of calcineurin signaling in axons that is critical for NGF-mediated growth, in a manner independent of transcription. A PxIxIT box present within specific dynamin1-splicing isoforms promotes interactions with calcineurin. Phosphoregulation of these PxIxIT-containing dynamin1 isoforms by NGF is required for TrkA internalization and axon growth. Together, our results point to a novel regulatory pathway by which NGF promotes axonal growth via calcineurin-mediated dephosphorylation of PxIxIT motif-containing dynamin1 isoforms and endocytosis of TrkA receptors.

RESULTS

Calcineurin Is Required for NGF-Mediated, but Not NT-3-Mediated, Sympathetic Axon Growth

To assess the role of calcineurin in neurotrophin-dependent sympathetic axon growth in vivo, we examined innervation of target tissues in mice with conditional ablation of calcineurin in neurons. Selective disruption of calcineurin in neurons was accomplished by crossing mice harboring a LoxP-based conditional *calcineurin* allele (*CaNB1$^{fl/fl}$* mice) (Zeng et al., 2001) to *Nestin-Cre* transgenic mice (Tronche et al., 1999). There are two mammalian isoforms of the calcineurin regulatory subunit, CalcineurinB; CaNB1 is ubiquitously expressed whereas CaNB2 is only expressed in testes. Immunoblotting analyses of sympathetic ganglia from *CaNB1$^{fl/fl}$;Nestin-Cre* mice showed reductions in the levels of CaNB1 and the calcineurinA catalytic subunit (CaNA) (see Figure S1A available online). A whole-mount tyrosine hydroxylase (TH) immunohistochemical assay was employed to visualize axonal growth out of sympathetic ganglia and innervation of several peripheral targets at late embryonic stages (E16.5–E18.5). TH immunostaining of E16.5 embryos revealed sympathetic fibers beginning to innervate the heart in both *CaNB1$^{fl/fl}$;Nestin-Cre* and wild-type littermates (Figures 1A–1D). However, in *CaNB1$^{fl/fl}$;Nestin-Cre* mutants, sympathetic axons were shorter and less branched (Figures 1B and 1D) as compared to that in wild-type embryos (Figures 1A and 1C). Deficits in sympathetic innervation were also observed in the dorsal face of the heart (Figures S1B–S1E), and in the kidneys (Figures S1F–S1I). At E18.5, although the main axonal fibers continued to elaborate into finer branches in the heart in wild-type mice (Figures 1E and 1G; Figures S1J and S1L), there were marked reductions in terminal extension and arborization of sympathetic fibers in *CaNB1$^{fl/fl}$;Nestin-Cre* mice (Figures 1F and 1H; Figures S1K and S1M). Similar deficits were observed in E18.5 salivary glands (Figures 1I–1L) and kidneys (Figures S1N–S1Q). In contrast to innervation deficits observed in final targets, axonal outgrowth from sympathetic ganglia (Figures 1M and 1N) and projections along the vasculature (Figures S1R and S1S) appeared normal in *CaNB1$^{fl/fl}$;Nestin-Cre* embryos. In addition, there were no differences in overall morphology of the sympathetic chain between mutant and wild-type embryos (Figures 1M and 1N). These results suggest that calcineurin is required for sympathetic innervation of final target tissues, an NGF-mediated process, but that axon growth along the vasculature, an NT-3-mediated process, occurs via calcineurin-independent mechanisms.

To directly test the requirement for calcineurin in promoting growth downstream of NGF and NT-3, we examined neurotrophin-dependent growth in compartmentalized cultures. In this culture system, neuronal cell bodies and axon terminals are segregated into distinct fluid compartments by a teflon-grease barrier (Figure 1O). Target-derived neurotrophins can be applied exclusively to axon terminals, recapitulating the in vivo situation. To genetically disrupt calcineurin activity in vitro, compartmentalized sympathetic cultures established from P0.5 *CaNB1$^{fl/fl}$* mice were infected with adenoviral vectors expressing either Cre recombinase or LacZ as control. Immunoblotting analyses showed significant reductions in the levels of CaNB and CaNA 48 hr after infecting *CaNB1$^{fl/fl}$* sympathetic neurons with Cre adenovirus (Figure S1T). *CaNB1$^{fl/fl}$* axons were then exposed to either NGF or NT-3, and growth was measured over 0–8 hr and 0–24 hr. NGF (100 ng/ml) supports approximately 60 μm of axon growth over 8 hr and 130 μm of axon growth over 24 hr (Figure 1T). Similar rates of axon growth were observed with NT-3 (100 ng/ml; 52 ± 6.4 μm and 117 ± 11.8 μm over 8 hr and 24 hr, respectively). Cre-mediated calcineurin depletion significantly decreased NGF-dependent axonal growth (Figures 1P, 1Q, and 1T). In contrast, NT-3-mediated axonal growth was not affected by the absence of calcineurin at 8 hr and largely was unaffected at 24 hr (Figures 1R, 1S, and 1T). Together with our in vivo results, these findings provide evidence that calcineurin activity in sympathetic neurons is required for axon growth in response to NGF, but not NT-3.

Calcineurin Signaling in Distal Axons Is Required for NGF-Mediated Axon Growth

Because target-derived NGF can activate calcineurin signaling either locally in axons or retrogradely in cell bodies, we asked whether calcineurin activity was required in cell bodies or in axons to promote axonal growth. Cell bodies or axons of rat sympathetic neurons grown in compartmentalized cultures were exposed to the calcineurin inhibitors Cyclosporin A (CsA) (2 μg/ml) and FK506 (0.2 μg/ml), and growth in response to axon-applied NGF (100 ng/ml) was assessed. As reported previously (Graef et al., 2003), pharmacological inhibition of calcineurin activity in neurons required the use of CsA and FK506 together because only partial inhibition was observed with either alone. NGF-dependent axon growth (Figures 2A and 2B) was markedly reduced when calcineurin inhibitors were added to distal axons (Figure 2C), but not cell bodies (Figures 2D). Decrease in NGF-dependent growth of axons exposed to calcineurin inhibitors was observed within 8 hr (Figure 2E), suggesting that calcineurin activity in axons is required for rapid axonal extension in response to NGF. Quantification revealed that calcineurin inhibition in distal axons significantly reduced NGF-dependent axonal growth by 51% over 8 hr and by 54% over 24 hr (Figure 2E). Consistent with our previous results, NT-3-dependent axon growth was not affected by the addition of CsA and FK506 to distal axons or cell bodies (Figures 2F–2J).

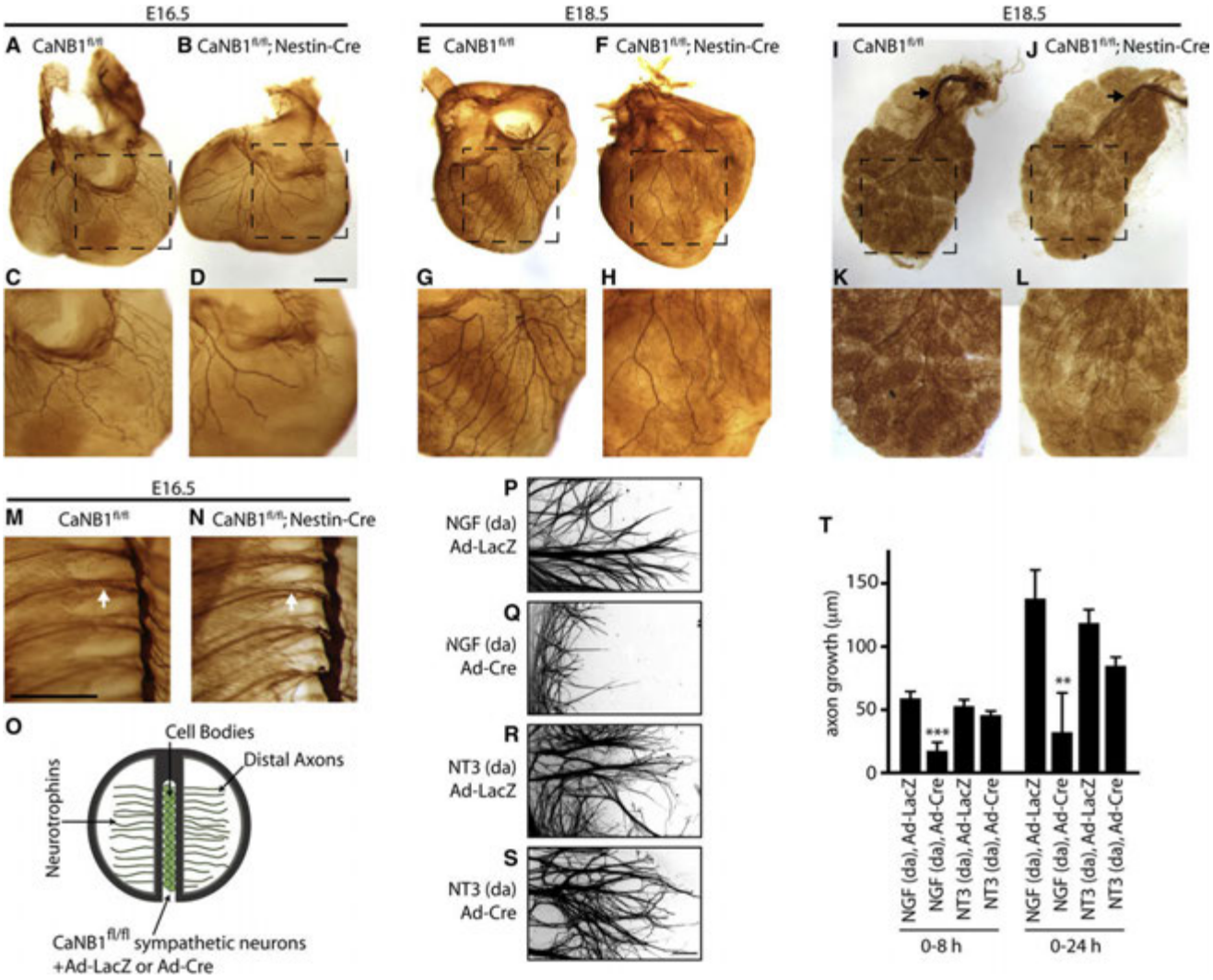


Figure 1. Calcineurin Is Required for NGF-Mediated, but Not NT-3-Mediated, Axon Growth in Sympathetic Neurons

(A–L) Whole-mount TH immunostaining shows reduced sympathetic fibers in target tissues in *CaNB1*$^{fl/fl}$;*Nestin-Cre* mice as compared to *CaNB1*$^{fl/fl}$ controls, at E16.5 (heart: A–D) and E18.5 (heart: E–H; salivary glands: I–L). Higher magnification images are shown in the lower panels. Black arrows (I and J) indicate sympathetic fibers approaching the salivary glands. Scale bar, 500 μm.

(M and N) There are no differences in sympathetic chain organization between E16.5 *CaNB1*$^{fl/fl}$ (M) and *CaNB1*$^{fl/fl}$;*Nestin-Cre* (N) mice. White arrow indicates TH-positive sympathetic fibers extending from sympathetic ganglia in both wild-type and mutant mice. Scale bar, 500 μm (n = 2 embryos for each genotype at E16.5, and at E18.5).

(O) *CaNB1*$^{fl/fl}$ sympathetic neurons were infected with adenoviral vectors expressing Cre (Ad-Cre) or LacZ (Ad-LacZ). Neurotrophins were added only to distal axons (da).

(P–S) Cre-mediated calcineurin deletion specifically decreases NGF-mediated, but not NT-3-mediated, axon growth. Axons were stained with β-III-tubulin for visualization after quantification of axon growth. Scale bar, 80 μm.

(T) Quantification of axon growth in compartmentalized cultures over 0–8 hr and 0–24 hr (**$p < 0.01$, ***$p < 0.001$). Results are mean ± SEM from n = 5 experiments.

Given that NGF-dependent, but not NT-3-dependent, axon growth requires calcineurin, we considered whether these two neurotrophins differ in their ability to activate calcineurin in sympathetic neurons. It is likely that neurotrophin signaling promotes activation of calcineurin through recruitment of PLC-γ to TrkA receptors (Graef et al., 2003) and the subsequent ability of PLC-γ to release Ca^{2+} from intracellular stores (Huang and Reichardt, 2003). To assess activation of the PLC-γ pathway in sympathetic neurons treated with either NGF (100 ng/ml) or NT-3 (100 ng/ml), we examined phosphorylation of TrkA at Tyr^{794}, previously identified as the PLC-γ binding site on rat TrkA (Loeb et al., 1994). Immunoblotting analyses with a phospho-specific antibody (Rajagopal et al., 2004) revealed that NGF increased TrkA phosphorylation at the PLC-γ interaction site (Y794), as compared to untreated control cultures, or cultures treated with NT-3 (Figures 2K and 2L). Recruitment of PLC-γ to TrkA upon neurotrophin stimulation leads to tyrosine phosphorylation of PLC-γ (Loeb et al., 1994), which is

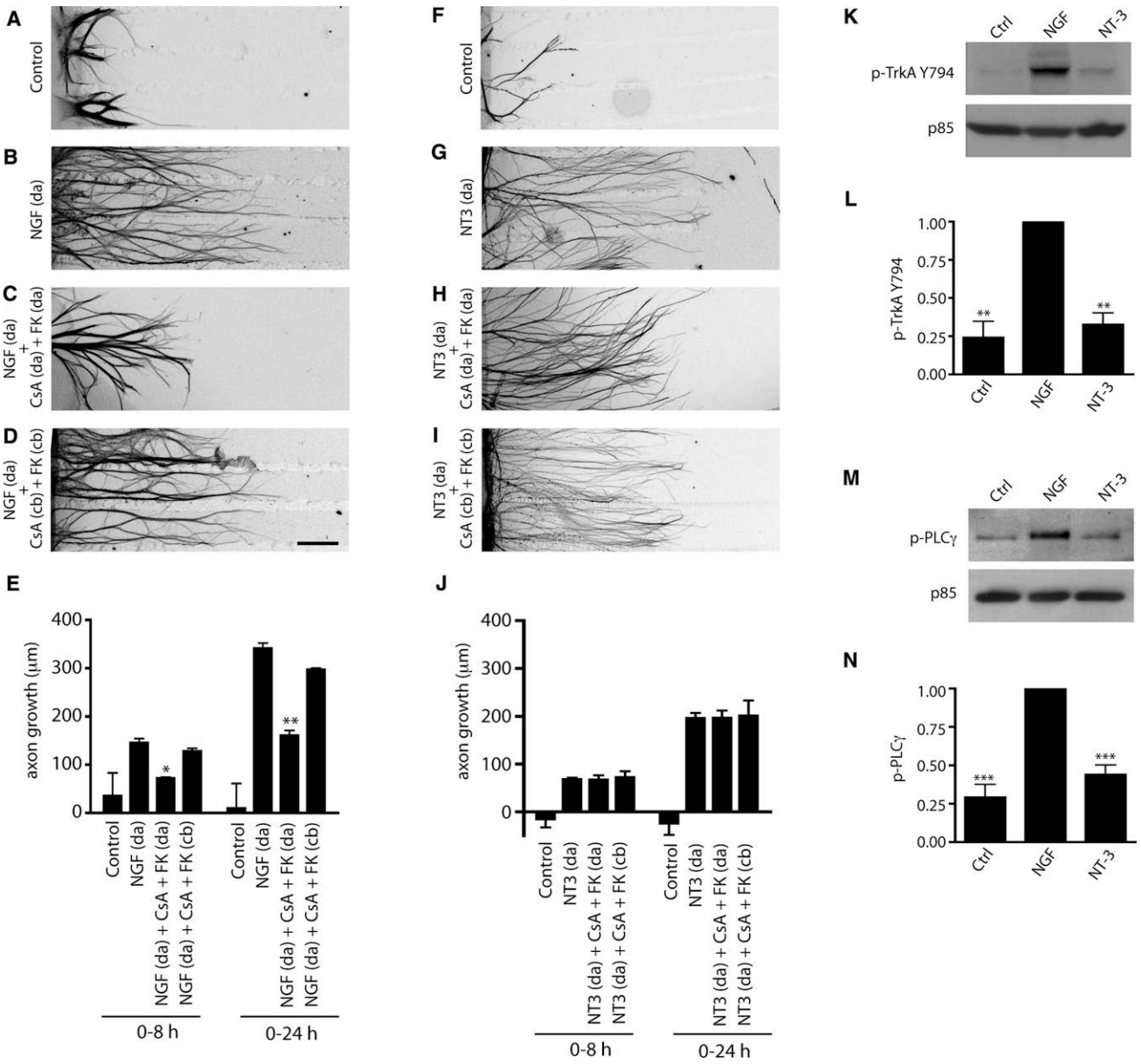


Figure 2. Calcineurin Signaling Is Required in Axons for NGF-Mediated Growth

(A–D) NGF-mediated axon growth is reduced by addition of calcineurin inhibitors (CsA+FK506) to distal axons (da) (C), but not cell bodies (cb) (D). NGF (100 ng/ml) was added only to distal axons. Axons were stained with β-III-tubulin for visualization. Scale bar, 320 μm.

(E) Quantification of NGF-mediated axon growth in compartmentalized cultures over 0–8 hr or 0–24 hr (*$p < 0.05$, **$p < 0.01$; n = 4 experiments).

(F–I) Calcineurin signaling is not required for NT-3-mediated axon growth.

(J) Quantification of NT-3-mediated axon growth (n = 4).

(K) NGF, but not NT-3, induces phosphorylation of TrkA on Tyr-794. Neuronal lysates were probed for phospho-TrkA (Y794). Immunoblots were reprobed for p85.

(L) Densitometric quantification of phospho-TrkA (Y794) (**$p < 0.01$; n = 3).

(M) NGF treatment selectively promotes tyrosine phosphorylation of PLC-γ. Lysates were immunoprecipitated with anti-phospho-tyrosine and probed for PLC-γ. Supernatants were probed for p85.

(N) Densitometric quantification of PLC-γ phosphorylation (***$p < 0.001$; n = 4).

a prerequisite step for activation of its enzymatic activity. We assessed PLC-γ tyrosine phosphorylation in untreated and NGF- and NT-3-treated sympathetic neuronal lysates by immunoprecipitating with an antibody directed against phosphotyrosine and probing immunoblots with a PLC-γ antibody. Only NGF treatment of sympathetic neurons resulted in enhanced tyrosine phosphorylation of PLC-γ (Figures 2M and 2N). These results suggest that selective activation of calcineurin by NGF in

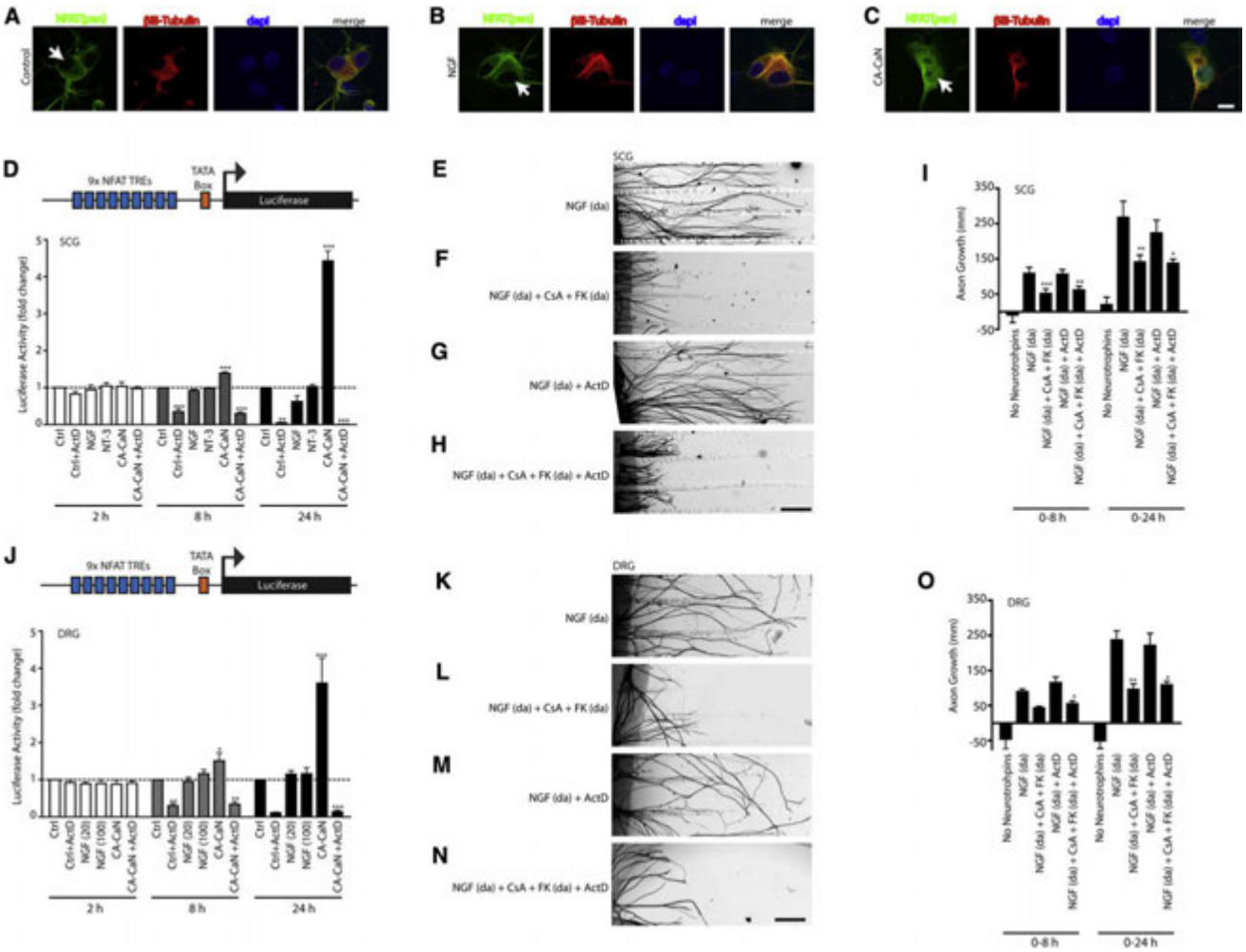


Figure 3. Calcineurin Supports NGF-Mediated Axon Growth in a Transcription-Independent Manner

(A–C) NGF does not promote nuclear import of NFAT transcription factors in sympathetic neurons. NFAT immunostaining shows that NGF treatment (100 ng/ml, 30 min) does not induce nuclear localization of NFAT (B, arrow), whereas NFAT nuclear labeling is evident in neurons expressing CA-CaN (C, arrow). Neurons were also immunostained with β-III Tubulin and DAPI. Scale bar, 10 μm.

(D) NFAT-luciferase reporter assay shows that NGF and NT-3 do not activate NFAT-dependent transcription in sympathetic neurons. Neurons expressing CA-CaN show activation of NFAT-dependent transcription at 8 and 24 hr after infection with NFAT-adenovirus, which is blocked by ActD (0.1 μg/ml) (**$p < 0.01$ and ***$p < 0.001$). Results are means ± SEM from three experiments.

(E–I) Calcineurin signaling is required for NGF-mediated axon growth in the absence of transcription. NGF and calcineurin inhibitors (CsA+FK506) were added only to distal axons. ActD (0.1 μg/ml) was bath applied. Scale bar, 320 μm.

(I) Quantification of sympathetic axon growth in compartmentalized cultures over 0–8 hr and 0–24 hr after treatments described in (E–H) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, n = 3 experiments).

(J) NFAT-luciferase assay shows that NGF does not activate NFAT-dependent transcription in DRG sensory neurons. DRG neurons expressing CA-CaN show robust activation of NFAT-dependent transcription (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, n = 5 experiments).

(K–N) Calcineurin signaling is required for NGF-mediated axon growth in DRG neurons, in the absence of transcription. Scale bar, 320 μm.

(O) Quantification of DRG axon growth in compartmentalized cultures (*$p < 0.05$, **$p < 0.01$, n = 4).

sympathetic neurons occurs via engagement of the PLC-γ signaling pathway.

NGF-Mediated Axonal Responses Require Calcineurin Signaling Independent of Transcriptional Activity

Previously, NFAT transcription factors were reported to be the major calcineurin substrate relevant for neurotrophin-mediated axon growth in developing sensory neurons (Graef et al., 2003). To test whether NGF stimulation promotes nuclear translocation of NFAT transcription factors in sympathetic neurons, we performed confocal microscopic analyses of neurons immunostained with a pan-NFAT antibody. Sympathetic neurons express all four Ca^{2+}-sensitive NFAT1-4 isoforms (Figure S2A). In unstimulated neurons, NFAT immunoreactivity was observed to be predominantly cytoplasmic, with staining observed both in cell bodies and axons including growth cones (Figure 3A; Figures

S2B and S2C). Importantly, exposure to NGF for 30 min failed to elicit any changes in NFAT subcellular localization (Figure 3B). However, expression of a constitutively active form of calcineurin (CA-CaN) that lacks the regulatory domain (De Windt et al., 2000) resulted in nuclear accumulation of NFAT (Figure 3C). As a more sensitive and quantitative assay, sympathetic neurons were infected with an adenoviral construct expressing an NFAT luciferase reporter containing nine multimerized NFAT binding sites upstream of a minimal TATA-containing promoter fused to luciferase (Figure 3D) (Wilkins et al., 2004). Exposure of neurons to NGF (100 ng/ml) for 2 hr, 8 hr, or 24 hr did not induce NFAT-dependent transcriptional activity (Figure 3D). Similarly, NT-3 had no effect on NFAT transcriptional activity (Figure 3D). However, adenovirus-mediated expression of constitutively active calcineurin increased luciferase reporter activity, at 8 hr and 24 hr after infection (1.4-fold and 4.4-fold at 8 hr and 24 hr, respectively), as compared to control untreated cultures (Figure 3D). Together, these results suggest that, at least over 24 hr, NGF does not induce activation of NFAT-dependent transcriptional activity in sympathetic neurons.

To determine whether calcineurin signaling mediates NGF-dependent axon growth via transcriptional responses, we directly tested the role of transcription in short-term axonal growth in response to NGF. Sympathetic neurons were grown in compartmentalized culture chambers, and transcriptional activity was blocked by adding ActinomycinD (ActD, 0.1 μg/ml). We confirmed that this concentration of ActD effectively blocked the ability of CA-CaN to induce NFAT-dependent transcription in sympathetic neurons (Figure 3D). ActD did not significantly influence NGF-dependent growth in sympathetic neurons over 8 hr or 24 hr (Figures 3E, 3G, and 3I). Similarly, treatment of sympathetic neurons with a different transcriptional inhibitor, α-amanitin (0.2 μg/ml), also had no effect on NGF-dependent axonal growth over 24 hr (Figure S2D). However, by 48 hr, we observed a complete cessation of NGF-mediated axonal growth with transcriptional inhibitors (Figure S2D), indicating that continued axonal growth after 24 hr requires new transcription. Importantly, application of the calcineurin inhibitors CsA and FK506 to axon terminals significantly reduced NGF-mediated axon growth over 24 hr, to 40%–60% of control values, in the presence (Figures 3H and 3I) or absence of ActD (Figures 3F and 3I). Together, these results provide evidence that calcineurin has a role in NGF-mediated axonal growth that is independent of transcription. Consistent with its role in transcriptionally independent NGF responses, calcineurin activity is required for rapid changes in growth cone morphology in response to NGF (Figures S2E–SI). NGF stimulation (15 min) leads to rapid increases in growth cone area (Figures S2F and S2H) and filopodia number (Figures S2F and S2I). These short-term effects of NGF are attenuated by calcineurin inhibition (Figures S2G–S1I).

To determine whether our findings extend to other NGF-responsive neuronal populations, we asked whether calcineurin has a transcription-independent growth-promoting effect in dorsal root ganglia (DRG) sensory neurons. Exposure of DRG neurons to NGF (20 ng/ml or 100 ng/ml) for 2 hr, 8 hr, or 24 hr did not induce NFAT-dependent transcriptional activity (Figure 3J), as reported by the NFAT-luciferase assay. However,

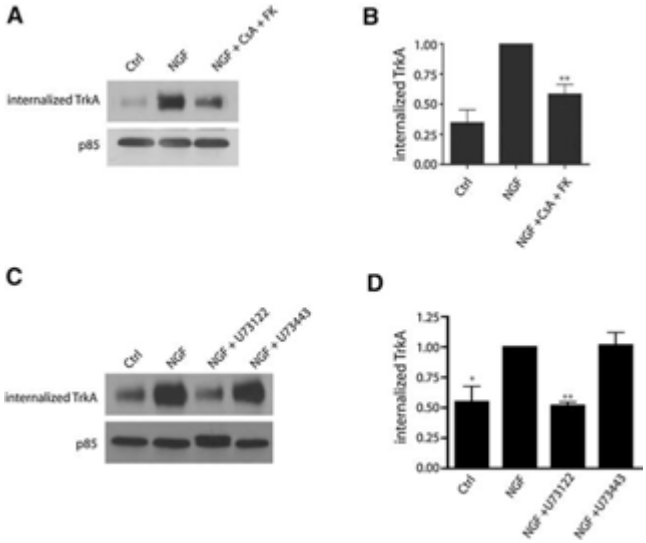


Figure 4. PLC-γ and Calcineurin Mediate TrkA Endocytosis

(A) Calcineurin activity is required for NGF-dependent internalization of TrkA receptors. Cell surface biotinylation and TrkA immunoblotting shows that NGF-dependent TrkA endocytosis is reduced by calcineurin inhibition.

(B) Densitometric quantification of internalized TrkA (**$p < 0.01$, $n = 6$).

(C) TrkA endocytosis is dependent on PLC-γ activity. PLC-γ inhibitor (U73122) decreases NGF-dependent TrkA endocytosis.

(D) Densitometric quantification of internalized TrkA (*$p < 0.05$ and **$p < 0.01$, $n = 4$). Supernatants were probed for p85 for normalization (A and C).

expression of constitutively active calcineurin increased luciferase reporter activity in DRG neurons (Figure 3J). Transcriptional inhibition did not significantly influence NGF-dependent growth in compartmentalized DRG cultures over 8 hr or 24 hr, but stopped axon growth by 48 hr (Figures 3K, 3M, and 3O; Figure S2J). Similar to our results with sympathetic neurons, application of the calcineurin inhibitors, CsA and FK506, to DRG axon terminals reduced NGF-mediated axon growth (40%–50% of control values) in the presence (Figures 3N and 3O) or absence of ActD (Figures 3L and 3O). Together, these results uncover a transcription-independent role for calcineurin in NGF-mediated axon growth in both sympathetic and DRG neurons.

PLC-γ/Calcineurin Signaling Promotes TrkA Endocytosis

The primary difference between NGF and NT-3 signaling in sympathetic neurons is that NGF is able to induce endocytosis of TrkA receptors whereas NT-3 cannot (Kuruvilla et al., 2004). Given that calcineurin signaling is required for NGF-dependent, but not NT-3-dependent, axonal responses, we hypothesized that calcineurin signaling might be required for NGF-mediated endocytosis of TrkA receptors. To test this hypothesis, a cell surface biotinylation assay was performed to measure NGF-dependent internalization of TrkA receptors in sympathetic neurons, in the presence or absence of calcineurin signaling. As previously reported (Kuruvilla et al., 2004), NGF treatment leads to robust internalization of TrkA receptors (Figures 4A and 4B). Levels of biotinylated TrkA receptors internalized in response to NGF were markedly reduced (42% decrease)

when calcineurin activity was blocked with CsA and FK506 (Figures 4A and 4B). Calcineurin inhibitors had no effect on surface levels of TrkA in the absence of NGF (Figures S3A and S3B), indicating that calcineurin signaling is not required for maintenance of TrkA receptors on the plasma membrane. Because our results suggest that NGF-mediated activation of calcineurin occurs via recruitment of the TrkA effector, PLC-γ, we tested whether PLC-γ activity is required for TrkA endocytosis. Inhibition of PLC-γ activity with a selective inhibitor, U73122 (10 μM), markedly reduced NGF-dependent endocytosis of TrkA receptors (Figures 4C and 4D). However, treatment of neurons with an inactive analog, U73443, had no effect. These results suggest that NGF promotes endocytosis of TrkA receptors by activation of a PLC-γ/calcineurin signaling pathway.

Calcineurin Mediates NGF-Dependent Growth via Dephosphorylation of Dynamin

Given that calcineurin signaling is required for TrkA endocytosis, we asked which calcineurin substrate mediates this response. Our clue came from previous studies in synaptic vesicle endocytosis (SVE), where calcineurin-dependent dephosphorylation of the endocytic GTPase dynamin1 is essential for the retrieval of synaptic vesicle membranes (Liu et al., 1994). Nerve terminal depolarization leads to calcineurin-dependent dephosphorylation of dynamin1 on at least two serine residues, Ser-774 and Ser-778, located within a phospho-box region in the proline-rich C terminus (Clayton et al., 2009). Site-directed mutagenesis indicated that phosphoregulation of these residues on dynamin1 is required for calcineurin-dependent endocytosis of synaptic vesicles (Clayton et al., 2009). To ask whether NGF stimulation leads to dynamin dephosphorylation in a calcineurin-dependent manner, sympathetic neurons were exposed to NGF for 20 min, and levels of phosphorylated dynamin1 were assessed using phospho-specific antibodies that specifically recognize dynamin1 phosphorylated on Ser-774 and Ser-778. NGF induced a significant decrease in dynamin1 phosphorylation on Ser-774 and Ser-778 (26.2% and 28.5%, respectively), which was blocked by CsA and FK506 treatment (Figures 5A and 5B). As predicted, the phosphorylation status of dynamin1 was unaffected by NT-3 treatment (Figures 5A and 5B). Thus, NGF, but not NT-3, leads to calcineurin-dependent dephosphorylation of dynamin, providing further support for this mechanism underlying the differential trafficking of TrkA receptors downstream of NGF and NT-3 (Kuruvilla et al., 2004).

Given that target-derived NGF acts directly on projecting axons to promote growth, we tested whether axon-applied NGF locally modulates dynamin1 phosphorylation in nerve terminals, in vitro, and in vivo. Sympathetic axons in compartmentalized cultures were stimulated with NGF (100 ng/ml, 20 min) and axonal lysates were immunoblotted with phospho-dynamin1 (Ser-778) antibody. Similar to results in mass cultures, NGF treatment of distal axons leads to a reduction (24% decrease) in phosphorylated dynamin1, in comparison to control treatment (Figure 5C and 5D). To test whether NGF regulates phosphorylation of dynamin1 in axons in vivo, we analyzed the levels of phospho-dynamin1 in a sympathetic target tissue, the salivary glands, in both wild-type and heterozygous *NGF* (*NGF*$^{+/-}$) mice. Given that dynamin1 is neuron specific (Urrutia et al., 1997), immunoblotting of salivary gland lysates with the phospho-dynamin1 antibody should reveal the status of dynamin1 phosphorylation locally in sympathetic nerve terminals that innervate the target tissue. If target-derived NGF regulates dynamin1 phosphorylation in vivo, then we would expect to see increased dynamin1 phosphorylation levels under conditions of reduced NGF signaling. We employed *NGF*$^{+/-}$ mice for this analysis because these mice display haploinsufficiency with reduced levels of NGF and sympathetic target innervation (Brennan et al., 1999; Ghasemlou et al., 2004), in contrast to homozygous *NGF* null mice, which completely lack sympathetic innervation (Glebova and Ginty, 2004). We found that *NGF*$^{+/-}$ mice have higher levels of phosphorylated dynamin1 on Ser-778 in sympathetic axons innervating the salivary glands, compared to wild-type animals (11.2% ± 2% increase; Figures 5E and 5F). These findings provide in vivo evidence for NGF-dependent phosphoregulation of dynamin1 locally in sympathetic axons.

To assess the role of dynamin1 dephosphorylation in supporting neurotrophin-dependent axon growth, sympathetic neurons were exposed for 24 hr to a cell-permeable peptide spanning the dynamin1 phospho-box (amino acids 769–784, incorporating Ser-774 and Ser-778) in which the two serines 774/778 were replaced with alanine (Ser774/778-Ala, dyn1$_{769\text{-}784AA}$). The dyn1$_{769\text{-}784AA}$ peptide blocks dephosphosphorylation-dependent dynamin1 functions by binding and sequestering downstream effector molecules, such as syndapin1 (Anggono et al., 2006). Delivery of dyn1$_{769\text{-}784AA}$ (300 μM) into sympathetic neurons reduced NGF-mediated axon growth from an average of 177 ± 14 μm/day to 90.6 ± 7.2 μm/day (Figures 5G, 5H, and 5M). In contrast, introduction of the phospho-mimetic peptide dyn1$_{769\text{-}784EE}$ (in which the serines 774/778 were substituted with glutamate) had no effect on NGF-mediated axon growth (Figures 5I and 5M). NT-3-mediated axon growth was not affected by delivery of either dyn1$_{769\text{-}784AA}$ or dyn1$_{769\text{-}784EE}$ (Figures 5J, 5K, 5L, and 5M). Together, these results provide evidence that calcineurin-mediated dephosphorylation of dynamin1 is a key signaling mechanism necessary for NGF-mediated, but not NT-3-mediated, axon growth.

Isoform-Specific Interaction of Calcineurin with Dynamin1 via a PxIxIT Domain

There are three *dynamin* genes expressed in mammals, with *dynamin1* reported to be neuron specific, *dynamin2* being ubiquitously expressed, and *dynamin3* expressed in brain, lungs, and testes (Urrutia et al., 1997). We asked whether calcineurin interacted with all three dynamins by performing calcineurinA-GST pull-down assays of rat brain lysates and probing for calcineurin interaction using antibodies specific to dynamin1, dynamin2, and dynamin3. As previously demonstrated (Lai et al., 1999), dynamin1 binds calcineurinA-GST (Figure S4A); in contrast, dynamin2 and dynamin3 do not detectably bind calcineurinA-GST (Figure S4A).

While exploring the mechanism of calcineurin-dynamin1 association, we observed that the dynamin1 C-terminal proline rich domain (PRD) harbors a putative calcineurin interaction sequence, PRITIS, within the amino acids 844–849 (Figure 6A). This motif has high sequence identity to the "PxIxIT box," a consensus sequence present in NFAT transcription factors that mediates the docking of calcineurin to the NFAT regulatory

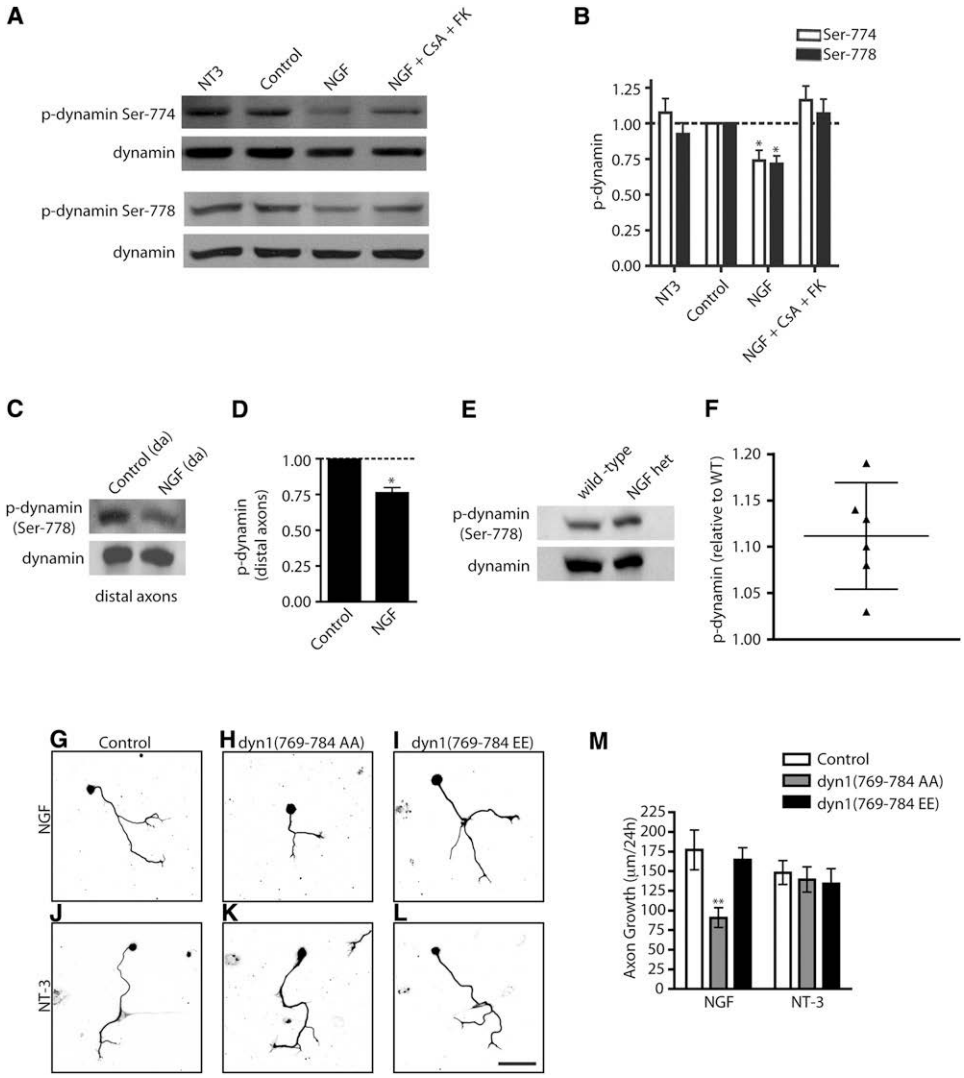


Figure 5. NGF Promotes Axon Growth through Dynamin Dephosphorylation

(A) NGF stimulation results in dephosphorylation of dynamin1 in a calcineurin-dependent manner. Neuronal lysates were immunoblotted using phospho-Ser774 and phospho-Ser778 dynamin antibodies. Immunoblots were stripped and reprobed for total dynamin1.

(B) Densitometric quantification of phospho-dynamin1 levels (*$p < 0.05$, n = 6).

(C) NGF stimulation results in dephosphorylation of dynamin1 (Ser 778) in distal axons. Immunoblots were reprobed for total dynamin1.

(D) Densitometric quantification of phospho-dynamin1 (Ser778) in axons (*$p < 0.05$, n = 3).

(E and F) $NGF^{+/-}$ mice have increased levels of phospho-dynamin1 in sympathetic axons in vivo. Salivary gland lysates from P0.5 wild-type and $NGF^{+/-}$ mice were immunoblotted using phospho-dynamin1 (Ser778) antibody. Immunoblots were reprobed for total dynamin1.

(F) Densitometric quantification of phospho-dynamin1 (Ser778) after treatments, as described in (E), is represented as a scatter plot with 95% confidence intervals. n = 6 pups for each genotype.

(G) Dephosphorylation-dependent dynamin1 function is required for NGF-mediated axon growth.

(H–L) Introduction of dyn1(769-784 AA) (H), but not the dyn1(769-784 EE) (I) peptide, decreased NGF-dependent axon growth over 24 hr. NT-3-mediated growth was unaffected by introduction of dyn1 phosphopeptides (J–L). Scale bar, 100 μm.

(M) Quantification of axon growth (**$p < 0.01$, n = 3).

domains (Aramburu et al., 1998). Deletion studies using a yeast-two hybrid assay had restricted the calcineurin-interaction region of dynamin1 to the last 135 amino acids at the C terminus (Lai et al., 1999), encompassing this putative PxIxIT box. To test whether calcineurin-dynamin1 interaction is mediated by the PxIxIT motif present in dynamin1, we took advantage of the VIVIT peptide, a high-affinity molecular mimic of the PxIxIT box domain that acts as a competitive inhibitor of calcineurin-PxIxIT box interactions (Aramburu et al., 1999). CalcineurinA-GST pull-down assays of rat brain lysates were performed either in the presence or absence of VIVIT, and immunoblotting was performed to detect dynamin1 interaction. The VIVIT peptide completely blocked calcineurin-dynamin1 interaction, whereas a control peptide, VEET, had no effect (Figure 6B).

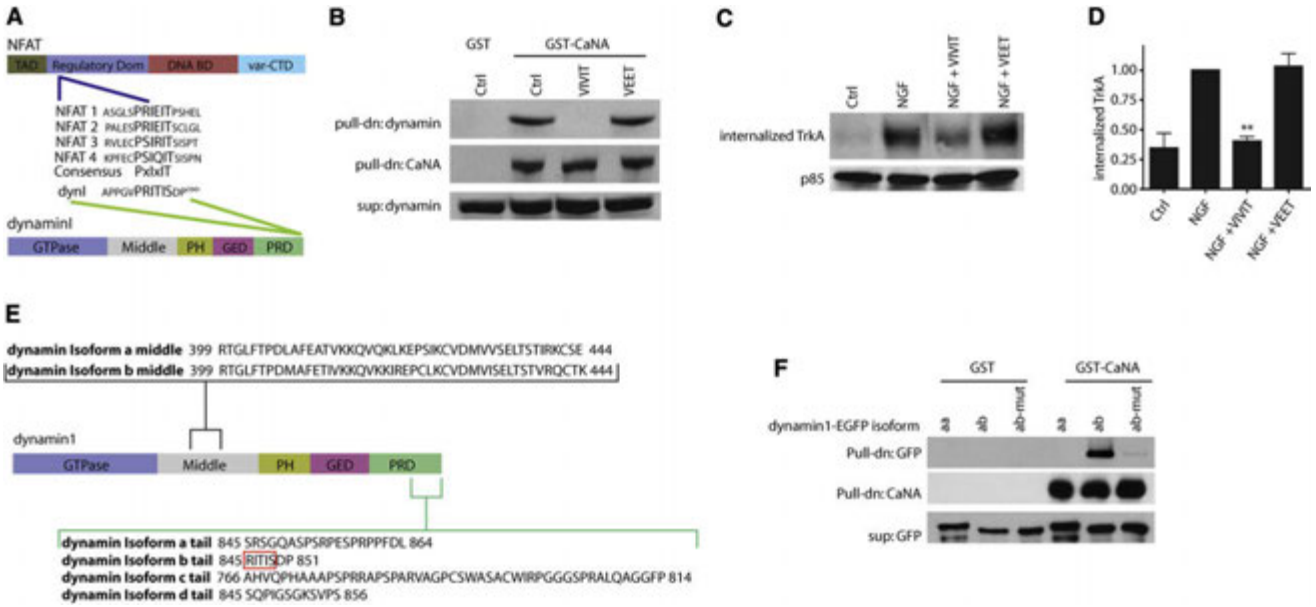


Figure 6. Calcineurin-Dynamin1 Interaction Is Mediated by a PxIxIT Motif Found in Specific Dynamin1 Isoforms

(A) Schematic of PxIxIT box consensus sequence found in the regulatory domain of NFAT (1–4) transcription factors and the PRITIS sequence in the proline-rich domain (PRD) of dynamin1. TAD is the transactivation domain, DNA BD is the DNA-binding domain, and var-CTD is the variable C-terminal domain for NFAT. PH is the pleckstrin homology domain, and GED is the GTPase effector domain for dynamin.

(B) Calcineurin-dynamin1 interaction is dependent on the PxIxIT motif. VIVIT peptide (a PxIxIT box mimic), but not a control VEET peptide, blocks association of CaNA with dynamin1. Pull-down with GST alone is shown as control.

(C) Calcineurin-dynamin1 interaction via the PxIxIT motif is required for NGF-dependent TrkA internalization. Cell surface biotinylation assay shows that VIVIT, but not VEET, treatment decreases NGF-dependent internalization of TrkA receptors. Supernatants were probed for p85.

(D) Densitometric quantification of internalized TrkA (**$p < 0.01$, $n = 4$).

(E and F) Calcineurin interaction is specific to dynamin1 variants with a PxIxIT box.

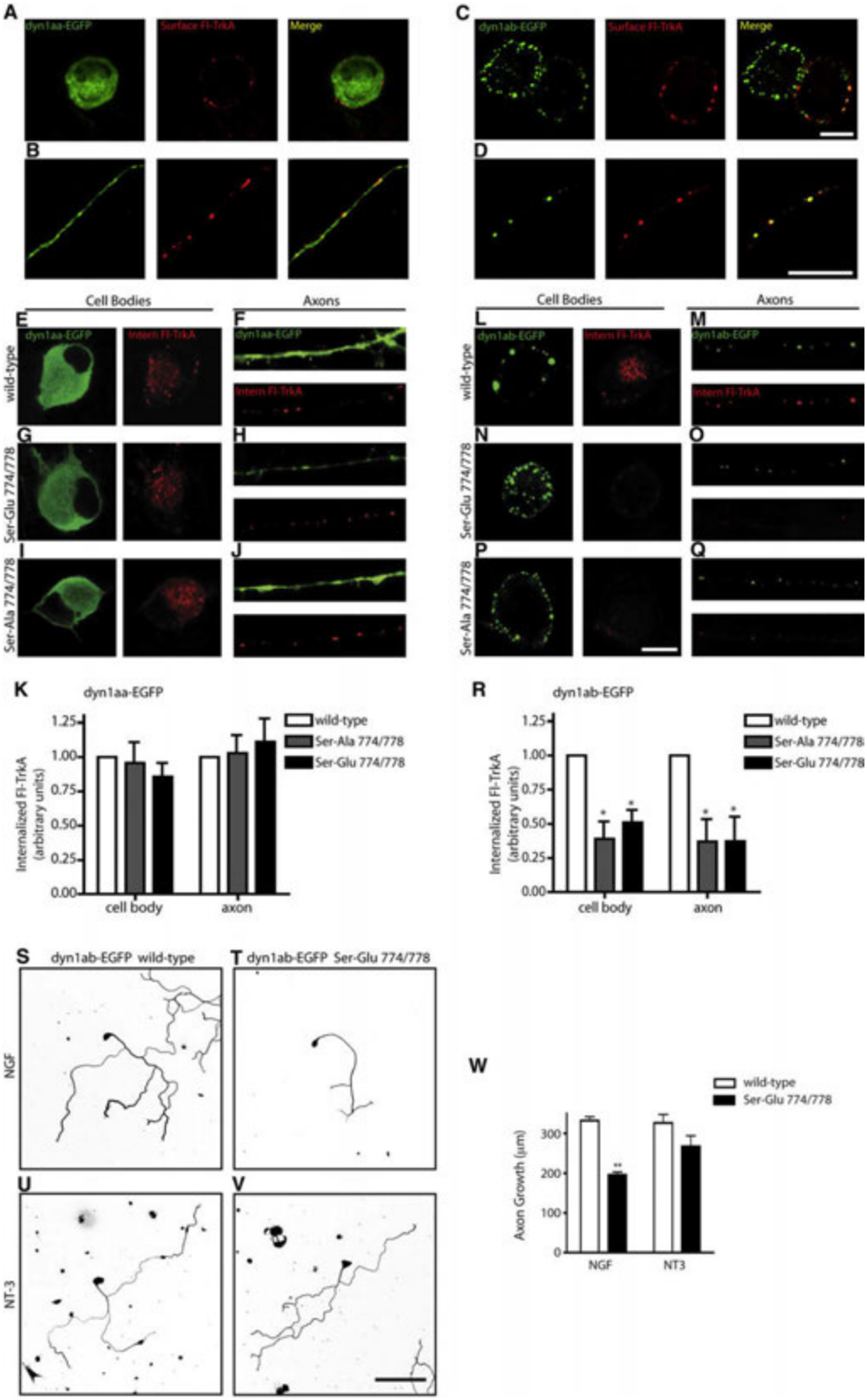
(E) Schematic of dynamin1 splicing variants. Red box indicates xIxIS portion of the PRITIS box sequence, which is only present in b tail isoforms.

(F) GST pull-down assays with HEK293 lysates show that calcineurin interacts with dynamin1ab via the PxIxIT box, but not dynamin1aa isoforms. HEK293 cells were transfected with dynamin1aa-EGFP, dynamin1ab-EGFP, or dynamin1ab-EGFP with PRITIS sequence mutated to ARATAA.

To investigate whether calcineurin signaling regulates TrkA endocytosis via its interaction with dynamin1, calcineurin-dynamin1 interaction was blocked by exposing cultured sympathetic neurons to a cell-permeable VIVIT peptide (1 μM), and a cell-surface biotinylation assay was performed to assess internalization of TrkA receptors in response to NGF. We observed that internalized TrkA levels following NGF treatment were significantly reduced (60% decrease) in the presence of VIVIT peptide, whereas application of the control peptide had no effect on TrkA internalization (Figures 6C and 6D). Treatment of sympathetic neurons with VIVIT or VEET did not significantly change the basal levels of surface TrkA receptors (Figures S4B and S4C). Thus, calcineurin association with dynamin1 via the PxIxIT box is required for NGF-dependent internalization of TrkA receptors.

Given that calcineurin-dynamin1 interaction is required for TrkA internalization, we asked whether this association is required for NGF-mediated axonal growth. Sympathetic neurons were grown in compartmentalized cultures, and axon growth in response to NGF was assessed over 24 hr. To disrupt calcineurin-dynamin1 interactions exclusively in cell bodies or distal axons, the VIVIT peptide was added either to cell body or axonal compartments. VIVIT application reduced axon growth only when added to distal axons, indicating that association of calcineurin with PxIxIT-containing proteins in axons is required for NGF-dependent growth (Figures S4D–S4G). VIVIT peptide did not disrupt NT-3-dependent axon growth (data not shown).

Although eight alternative spliced isoforms of dynamin1 are expressed in neurons (Cao et al., 1998), only two isoforms contain the PxIxIT motif. Dynamin1 contains two splicing regions; use of the first splicing region results in two isoforms of equal size but different nucleotide sequences (a and b forms). Additionally, there are at least four splicing variants of the C-terminal region, resulting in four distinct tail regions: a, b, c, and d (Figure 6E). Thus, dynamin1 has at least eight spliced variants: aa, ba, ab, bb, ac, bc, ad, and bd (Cao et al., 1998). Interestingly, only splice variants with the b tail region (ab and bb) contain the PxIxIT box motif (Figure 6E). To test whether dynamin1 splice variants bearing the b tail specifically interact with calcineurin, dynamin1aa (without PxIxIT) and dynamin1ab (with PxIxIT) were tagged with EGFP and expressed in HEK293 cells. Cell lysates were tested for interaction of the specific dynamin1 isoforms with calcineurinA-GST in pull-down assays. As predicted, dynamin1ab isoform that contains the PxIxIT box interacted with calcineurinA-GST. Neither the dynamin1aa isoform nor a dynamin1ab isoform with a mutated PxIxIT box (PRITIS → ARATAA) was able to bind calcineurinA-GST in pull-down assays (Figure 6F).


A
dyn1aa-EGFP
Surface FI-TrkA
Merge
B
C
dyn1ab-EGFP
Surface FI-TrkA
Merge
D
Cell Bodies
Axons
E
dyn1aa-EGFP
Intern FI-TrkA
wild-type
F
dyn1aa-EGFP
Intern FI-TrkA
G
Ser-Glu 774/778
H
I
Ser-Ala 774/778
J
L
dyn1ab-EGFP
Intern FI-TrkA
wild-type
M
dyn1ab-EGFP
Intern FI-TrkA
N
Ser-Glu 774/778
O
P
Ser-Ala 774/778
Q
K
dyn1aa-EGFP
Internalized FI-TrkA (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
wild-type
Ser-Ala 774/778
Ser-Glu 774/778
cell body
axon
R
dyn1ab-EGFP
Internalized FI-TrkA (arbitrary units)
1.25
1.00
0.75
0.50
0.25
0.00
wild-type
Ser-Ala 774/778
Ser-Glu 774/778
cell body
axon
S
dyn1ab-EGFP wild-type
T
dyn1ab-EGFP Ser-Glu 774/778
NGF
U
V
NT-3
W
wild-type
Ser-Glu 774/778
Axon Growth (µm)
300
200
100
0
NGF
NT3

Phosphoregulation of PxIxIT-Containing Dynamin1 Isoforms Is Required for TrkA Endocytosis and Axon Growth

Different dynamin1 splicing isoforms display different subcellular localization in heterologous expression systems (Cao et al., 1998). To examine the subcellular localization of dynamin1aa and dynamin1ab isoforms, sympathetic neurons were electroporated with vectors expressing EGFP-tagged dynamin1aa or dynamin1ab. The two dynamin isoforms showed striking differences in subcellular localization in sympathetic neurons. Dynamin1aa-EGFP showed diffuse cytoplasmic localization throughout the cell bodies (Figure 7A) and axons (Figure 7B). In stark contrast, dynamin1ab-EGFP expression resulted in a punctate staining along the plasma membrane and throughout the cytoplasm in cell bodies (Figure 7C) and axons (Figure 7D). Because the amino acid sequences of these two isoforms differ only in the C-terminal region containing the PxIxIT motif (Figure 6E), these results indicate that relatively small differences in primary sequence can result in striking changes in cellular localization. We next examined whether the punctate distribution of dynamin1ab-EGFP colocalizes with surface TrkA receptors. To visualize surface TrkA receptors, a live-cell antibody feeding assay was performed in sympathetic neurons (Ascano et al., 2009) expressing both N-terminal FLAG-tagged TrkA receptors and dynamin1-EGFP isoforms. Using an antibody directed against the extracellular FLAG epitope of the TrkA receptors, we found that the punctate distribution of dynamin1ab-EGFP fluorescence at the plasma membrane was in close apposition to surface TrkA immunoreactivity in neuronal soma (Figure 7C) and axons (Figure 7D), under nonpermeabilizing conditions. In contrast, little colocalization between surface TrkA labeling and dynamin1aa-EGFP was observed (Figures 7A and 7B). Together, these results suggest that dynamin1ab isoforms might mediate TrkA endocytosis in sympathetic neurons.

To test whether phosphoregulation of dynamin1 is critical for NGF-dependent endocytosis of TrkA receptors, we generated phosphomutants of the dynamin1aa and dynamin1ab isoforms. Because NGF stimulation results in dephosphorylation of dynamin1 on Ser 774 and 778, we generated dynamin1aa and dynamin1ab mutants bearing mutations of both serine residues to either alanine (Ser774/778-Ala; nonphosphorylatable forms) or glutamate (Ser774/778-Glu; phosphomimetic forms). Previous studies had shown that both the nonphosphorylatable and phosphomimetic forms of dynamin1 act as dominant negative inhibitors of activity-dependent synaptic vesicle endocytosis (Anggono et al., 2006; Clayton et al., 2009).

To label and follow endocytic trafficking of surface TrkA receptors, sympathetic neurons coexpressing FLAG-TrkA and the dynamin1 constructs were live-labeled with a calcium-sensitive FLAG antibody. After exposure to NGF for 30 min to allow internalization of labeled receptors, surface-bound antibodies were stripped, leaving antibodies bound only to the internalized pool of receptors. FLAG antibodies bound to internalized receptors were then visualized with Alexa-546-labeled secondary antibodies. We observed robust internalization of TrkA receptors in cell bodies and axons in response to NGF stimulation in cells expressing wild-type (Figures 7E and 7F), phosphomimic (Ser774/778 to Glu) (Figures 7G and 7H), or phosphomutant (Ser774/778 to Ala) (Figures 7I and 7J) dynamin1aa-EGFP. In contrast, expression of either dynamin1ab-EGFP phosphomimetic mutant (Ser774/778-Glu) (Figure 7N) or the nonphosphorylatable dynamin1ab-EGFP mutant (Ser774/778-Ala) (Figure 7P) significantly reduced NGF-mediated TrkA internalization in cell bodies to 39% and 50%, respectively, when compared to neurons expressing wild-type dynamin1ab-EGFP (Figures 7L and 7R). Expression of both phosphomutant forms of dynamin1ab-EGFP similarly reduced NGF-dependent internalization in axons (63% decrease) (Figures 7M, 7O, 7Q, and 7R). Expression of mutant dynamin1ab-EGFP forms did not affect surface expression of FLAG-TrkA receptors in the absence of NGF treatment, nor did it influence the ability of FLAG antibodies to bind surface receptors (Figures S5A–S5C), indicating that decreased intracellular accumulation of FLAG-TrkA in mutant dynamin1ab-expressing cells indeed reflects a block in endocytosis. The finding that both the nonphosphorylatable (Ser-Ala) and phosphomimetic (Ser-Glu) mutations inhibited receptor internalization suggests that, similar to synaptic vesicle endocytosis (Anggono et al., 2006), the cycle between dynamin phosphorylation and dephosphorylation is critical for NGF-dependent endocytosis of TrkA receptors. Together, these results indicate that NGF promotes internalization of its receptors through calcineurin-mediated dephosphorylation of specific spliced variants of dynamin1 harboring a PxIxIT interaction motif.

To determine whether phosphoregulation of dynamin1ab isoforms is also important for NGF-dependent axon growth, we infected sympathetic neurons with adenoviruses expressing

Figure 7. Phosphoregulation of Dynamin1ab Is Required for TrkA Endocytosis and Axon Growth

(A–D) Dynamin1aa isoform (A and B) shows diffuse cytoplasmic localization, whereas dynamin1ab isoform (C and D) shows punctate localization in cell bodies and axons. FLAG immunostaining shows surface FLAG-TrkA receptors in cell bodies (A and C) and in axons (B and D). Scale bar, 10 μm.

(E–J) Phosphoregulation of dynamin1aa is not required for NGF-dependent TrkA internalization. Neurons were transfected with FLAG-TrkA and either wild-type dynamin1aa-EGFP (E and F), Ser-Glu 774/778 dynamin1aa-EGFP (G and H), or Ser-Ala 774/778 dynamin1aa-EGFP (I and J). Cell bodies are shown in (E), (G), and (I). Axons are shown in (F), (H), and (J). Scale bar, 10 μm.

(K) Quantification of NGF-dependent TrkA internalization in cell bodies and axons (n = 3).

(L–Q) Phosphomutants of dynamin1ab disrupt NGF-dependent internalization of TrkA. Neurons were transfected with FLAG-TrkA and either wild-type dynamin1ab-EGFP (L and M), Ser-Glu 774/778 dynamin1ab-EGFP (N and O), or Ser-Ala 774/778 dynamin1ab-EGFP (P and Q). Cell bodies are shown in (L), (N), and (P). Axons are shown in (M), (O), and (Q). Scale bar, 10 μm.

(R) Quantification of internalized TrkA (*$p < 0.01$, n = 3).

(S–V) Phosphoregulation of dynamin1ab is required for NGF-dependent, but not NT-3-dependent, axon growth. NGF-mediated growth is blocked in sympathetic neurons expressing dynamin1ab-EGFP Ser-Glu 774/778 (T) as compared to wild-type dynamin1ab-EGFP (S). NT-3-mediated growth was unaffected (U and V). Scale bar, 50 μm.

(W) Quantification of neurite length (**$p < 0.01$, n = 3).

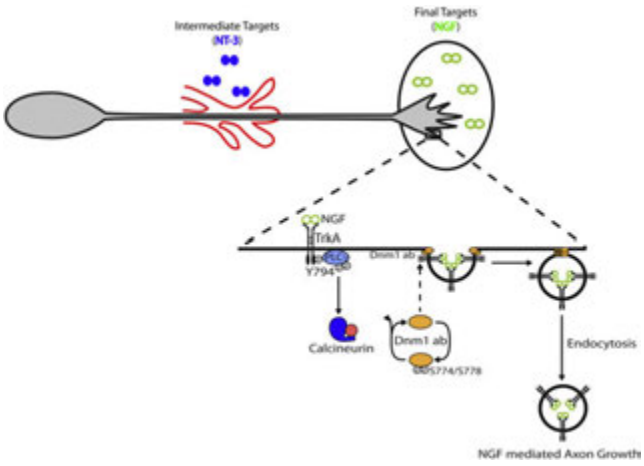


Figure 8. NGF and NT-3 Differentially Employ Calcineurin Signaling to Promote Axonal Growth in Sympathetic Neurons

NT-3 and NGF act upon a common TrkA receptor in sympathetic neurons to regulate distinct stages of axonal growth. NT-3 promotes proximal axon extension along the vasculature, whereas NGF is required for innervation of final peripheral targets. NGF-specific engagement of the PLC-γ effector pathway activates the calcium-responsive phosphatase, calcineurin. Calcineurin dephosphorylates PxIxIT motif-containing dynamin1 isoforms to promote TrkA endocytosis. Calcineurin-mediated endocytic trafficking of TrkA receptors signals locally in sympathetic nerve terminals to promote NGF-dependent axonal extension and branching within final target tissues.

either wild-type dynamin1ab-EGFP or the phosphomimetic dynamin1ab-EGFP mutant (Ser774/778-Glu) and measured axon growth in response to NGF over 36 hr. Expression of the mutant dynamin1ab-EGFP significantly reduced NGF-dependent neurite outgrowth, compared to that in neurons expressing wild-type dynamin1ab-EGFP (Figures 7S and 7T). When quantified, the longest neurite in dynamin1ab-EGFP mutant (Ser774/778-Glu)-expressing cells was, on average, 136 μm shorter than the axons of control cells (195.8 ± 7 μm in dynamin1ab-EGFP Ser774/778-Glu-expressing neurons versus 332.2 ± 9.2 μm in wild-type dynamin1ab-EGFP neurons) (Figure 7W). Consistent with our previous results, NT-3 mediated axon growth was not significantly affected by the dynamin1ab phosphomutant (Figures 7U, 7V, and 7W). Together, these results suggest that phosphoregulation of PxIxIT box-containing dynamin1 isoforms is necessary to mediate NGF-dependent endocytosis of TrkA receptors and axonal growth.

DISCUSSION

Endocytosis of NGF and its receptor, TrkA, in developing neurons provides one of the best examples of the significance of growth factor receptor trafficking in neurobiology. However, surprisingly little is known of the mechanisms by which NGF signaling modulates the core endocytic machinery to promote its own trafficking, and even less is known about the role of endocytosis in local NGF-promoted growth events in axon terminals. Here, we identify calcineurin-mediated dephosphorylation of dynamin1 as a mechanism by which target-derived NGF promotes internalization of its TrkA receptors and axon growth (Figure 8). We show that specific spliced variants of dynamin1 interact with calcineurin and that phosphoregulation of only these isoforms mediates neurotrophin receptor endocytosis and axon growth. Importantly, our results point to critical differences in the mechanisms by which two neurotrophins, NT-3 and NGF, act on a common TrkA receptor to promote proximal and distal stages of axonal growth during sympathetic nervous system development, with NGF-dependent growth showing a selective need for calcineurin-mediated endocytic events locally in nerve terminals.

Previously, transcriptional programs controlled by calcineurin have been demonstrated to be critical for axonal growth, dendritic structure, and synapse formation (Flavell et al., 2006; Graef et al., 2003; Schwartz et al., 2009). Our results uncover a new transcription-independent mechanism by which calcineurin mediates neuronal responses to extrinsic neurotrophic cues. We found that, over 24 hr, axon growth in response to NGF acting locally at axon terminals in sympathetic and DRG sensory neurons was significantly attenuated by calcineurin inhibition but not transcriptional blockade. Thus, we favor the hypothesis that calcineurin-mediated TrkA trafficking influences early growth events through local axonal mechanisms. Currently, it remains unclear as to why TrkA endocytosis might be selectively required for NGF-mediated, but not NT-3-mediated, axonal growth in sympathetic neurons. One possible explanation might be that, because NGF uniquely promotes TrkA endocytosis in nerve terminals for carrying retrograde survival signals back to neuronal soma, this process has been co-opted for local control of NGF-mediated axonal growth, via mechanisms that remain to be identified. It is possible that TrkA localization to endocytic vesicles might enhance downstream signaling, perhaps by prolonging association with downstream signaling effectors, spatially concentrating activated receptors, or by recycling receptors back to the membrane for repeated interaction with ligand.

Our findings that NGF does not induce NFAT activation within 24 hr in sympathetic and DRG sensory neurons do not preclude a requirement for calcineurin/NFAT-mediated transcriptional activity in supporting long-term axonal growth. Although we found that transcriptional activity is not required for NGF-mediated axonal growth over the first 24 hr, continued axonal growth after 24 hr requires new gene expression. This may reflect a specific role for NGF-mediated transcriptional responses, acting either via the calcineurin/NFAT, MAPK/SRF (Wickramasinghe et al., 2008), or CREB pathways (Lonze et al., 2002). Alternatively, this may reflect a general loss of proteins important for axonal growth during the extended treatments with transcriptional inhibitors. Together with the previously published study by Graef et al. (2003), our findings might reflect a biphasic mechanism of action for calcineurin in neurotrophin-mediated axonal growth. Thus, calcineurin might act early, via trafficking of TrkA receptors in axons and local activation of growth-promoting pathways, and at later stages, via activation of NFAT-mediated transcription. *NFATc2/c3/c4* triple null mice die early, at embryonic day E11.5 (Graef et al., 2001), prior to the formation of sympathetic axons and innervation of target tissues. Further studies using mice with conditional deletion of *NFAT* isoforms will be needed to elucidate the contribution of NFAT-mediated transcription to the developing sympathetic

nervous system. Nevertheless, our results indicate that NFAT transcription factors are not the sole targets of calcineurin relevant for neurotrophin-mediated axon growth. Our identification of a novel endocytic mechanism by which calcineurin signaling promotes neurotrophin-dependent axonal growth provides insight into the versatility of calcineurin signaling in nervous system development and, in particular, neurotrophin-mediated functions.

Our study suggests parallels between neurotrophin receptor endocytosis in developing neurons and local synaptic vesicle recycling in mature nerve terminals. During synaptic vesicle endocytosis, dynamin1 is among a group of structurally distinct proteins collectively called dephosphins that undergo a cycle of dephosphorylation and rephosphorylation in nerve terminals to mediate synaptic vesicle recycling and synaptic transmission (Cousin and Robinson, 2001). We show that calcineurin-dependent dephosphorylation of dynamin1 is a common mechanism underlying TrkA and synaptic vesicle endocytosis. Neurotrophins modulate synaptic transmission in mature neurons (Lu, 2004), and our results suggest that a potential target for neurotrophin actions at presynaptic terminals might be the regulation of calcineurin-dynamin1-dependent retrieval of synaptic vesicles after exocytosis.

Alternative splicing of the three *dynamin* genes generates over 25 different variants (Cao et al., 1998) that could greatly increase the diversity of dynamin functions in the mammalian nervous system. We provide evidence that specific dynamin1-splicing isoforms exhibit distinct subcellular localizations in neurons and perform discrete biological functions. In addition to synaptic vesicle retrieval, calcineurin-dynamin1-mediated endocytosis has been shown to be critical for regulation of AMPA receptor densities at postsynaptic spines during paradigms of synaptic plasticity, such as long-term depression (LTD) (Beattie et al., 2000; Lin et al., 2000). Our findings raise the possibility that PxIxIT-containing dynamin1 isoforms might mediate all other calcineurin-regulated endocytosis in neurons.

The role of NGF-dependent regulation of calcineurin in endocytosis and axon outgrowth may have implications that extend beyond early neural development to the pathogenesis of some neurodegenerative disorders. Defective NGF trafficking in basal forebrain cholinergic neurons has been implicated in degeneration and atrophy of these neurons in Down's syndrome and Alzheimer's disease (Cooper et al., 2001; Salehi et al., 2006). Overexpression of *Regulator of Calcineurin 1* (*RCAN1*) encoding for an endogenous calcineurin inhibitor has also been implicated in neuropathology of Down's syndrome and Alzheimer's disease (Ermak et al., 2001; Fuentes et al., 2000). In future experiments, it will be intriguing to investigate the role of regulated calcineurin-dependent endocytosis in the trafficking of TrkA receptors and in maintaining the integrity of basal forebrain cholinergic neurons in normal and diseased states.

EXPERIMENTAL PROCEDURES

Animals

To generate conditional mutants of *CaNB1*, floxed *CαNB1* (*CaNB1*$^{fl/fl}$) mice (Jackson Laboratory) were crossed to *Nestin-Cre* mice (Jackson Laboratory). *NGF*$^{+/-}$ mice (Crowley et al., 1994) were obtained from the Jackson Laboratory. All procedures relating to animal care and treatment conformed to institutional and NIH guidelines.

Immunostaining

Whole-mount tyrosine hydroxylase immunohistochemistry was performed on E16.5–E18.5 mouse embryos, as previously described (Kuruvilla et al., 2004). For NFAT immunostaining, sympathetic neurons were treated with 100 ng/ml NGF for 30 min, and neurons were fixed and immunostained using pan-NFAT antibody, β-III-tubulin, and DAPI (4′,6-diamidino-2-phenylindole). Images representing 1 μm optical slices were acquired using a Zeiss LSM 510 confocal scanning microscope equipped with diode (405 nm), Ar (458–488 nm), and He/Ne (543–633) lasers.

Neuronal Cultures

Sympathetic neurons were harvested from P0.5 Sprague-Dawley rats and were grown in mass cultures or compartmentalized cultures, as described previously (Kuruvilla et al., 2004). Dissociated DRG neurons were isolated from E15–16 rats and were grown in mass cultures or compartmentalized cultures, using culture conditions similar to that described for sympathetic neurons. Plasmids, adenoviral vectors, pharmacological reagents, and antibodies used in this study are described in detail in Supplemental Experimental Procedures.

Axon Growth Assays

Axon growth in compartmentalized cultures was quantified by capturing phase contrast images of the distal axon compartments over 8 hr or consecutive 24 hr intervals using a Zeiss Axiovert 200 microscope with a Retiga EXi camera. Rate of axonal growth (μm/day) was measured using Openlab 4.04. For all neurite growth assays in mass cultures, images were taken using an Axio Imager M1 (Zeiss) microscope, and length of the longest neurite was measured using Axiovision software (Zeiss). Measurements from 30 to 50 neurons were averaged for each condition for a single experiment. Details of analyses of neurotrophin-dependent neurite growth with dynamin1 phosphopeptides and short-term changes in growth cone morphologies are described in Supplemental Experimental Procedures.

Luciferase Reporter Assays

Sympathetic neurons were infected with NFAT-luciferase reporter adenovirus for 24 hr, and then neurons were stimulated with control media, NGF, or NT-3 (100 ng/ml) for 2, 8, and 24 hr; reporter gene activity was assessed with Luciferase Reporter Assay System (Promega, E1910). Similar analyses were used to report NFAT transcriptional activity in DRG neurons.

TrkA Receptor Internalization Assays

Cell-surface biotinylation assays were performed in cultured sympathetic neurons as previously described (Kuruvilla et al., 2004). Live cell antibody feeding assays were performed as previously described (Ascano et al., 2009).

Immunoblotting, Immunoprecipitation, and Pull-Down Assays

For analysis of tyrosine phosphorylation of PLC-γ, sympathetic neurons were treated with NGF or NT-3 (100 ng/ml) for 30 min at 37°C. Cells were lysed with RIPA solution, and lysates were subjected to immunoprecipitation with anti-phosphotyrosine (PY-20; Sigma) and were incubated with Protein-A agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were then immunoblotted for PLC-γ. To detect phosphorylated dynamin1 in sympathetic axons in vitro, sympathetic neurons grown in compartmentalized cultures were stimulated with NGF applied to distal axons for 20 min or were treated with control medium; axonal lysates were prepared and subjected to immunoblotting with the phospho-dynamin1 (Ser 778) antibody. To assess dynamin1 phosphorylation in sympathetic nerve terminals in vivo, salivary glands harvested from P0.5 wild-type and *NGF*$^{+/-}$ mice were subjected to immunoblotting with the phospho-dynamin1 (Ser 778) antibody. All immunoblots were visualized with ECL Plus Detection Reagent (GE Healthcare) and were scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare). For pull-down assays, CalcineurinA-GST recombinant protein expression was induced with 100 μM IPTG for 4–6 hr at 25°C. Calcineurin-GST protein was immunoprecipitated from bacterial cell lysates with 500 μl of 50% glutathione-agarose. CalcineurinA-GST was resuspended in PBS plus phenylmethanesulphonylfluoride (PMSF, 1 mM) plus sodium azide (10 μM). P0.5 rat brain (1 g) was homogenized in calcium-containing lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2% Triton

X-100, 0.5 mM B-mercaptoethanol, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, and 10 μM sodium azide) and centrifuged. Calcineurin-GST pull-down assays of rat brain lysates were performed at 4°C for 1 hr. A similar protocol was used for Calcineurin-GST pull-down assays from HEK293 lysates.

Statistical Analyses

InStat software was used for statistical analyses. All Student's t tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.04.025.

ACKNOWLEDGMENTS

We thank Antonella Riccio, Samer Hattar, and Haiqing Zhao for insightful comments on this manuscript. We thank Mark McNiven for providing dynamin1 constructs, Moses Chao for the P-TrkA (Y794) antibody, and Lois Greene for the adenovirus-Cre. This work was supported by US National Institutes of Health (grant R01 MH080738) and a Whitehall Foundation award to R.K.

Accepted: April 7, 2011
Published: June 22, 2011

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