PI-3 kinase and IP3 are both necessary and sufficient to mediate NT3-induced synaptic potentiation

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Signaling mechanisms underlying neurotrophic regulation of synaptic transmission are not fully understood. Here we show that neurotrophin-3 (NT3)-induced potentiation of synaptic transmission at the neuromuscular synapses is blocked by inhibition of phosphoinositide-3 kinase, phospholipase C-γ or the downstream IP3 receptors of phospholipase C-γ, but not by inhibition of MAP kinase. However, neither stimulation of Ca^{2+} release from intracellular stores by photolysis of caged IP3, nor expression of a constitutively active phosphoinositide-3 kinase (PI3K*) in presynaptic motoneurons alone is sufficient to enhance transmission. Photo-uncaging of IP3 in neurons expressing PI3K* elicits a marked synaptic potentiation, mimicking the NT3 effect. These results reveal an involvement of PI3 kinase in transmitter release, and suggest that concomitant activation of PI3 kinase and IP3 receptors is both necessary and sufficient to mediate the NT3-induced synaptic potentiation.

Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT3 and NT4/5, are a family of structurally and functionally related proteins that regulate neuronal survival and differentiation[1]. Studies have revealed an acute modulation of synaptic transmission and plasticity by neurotrophins (for review, see refs. 2 and 3). For example, application of exogenous BDNF or NT3 rapidly enhances synaptic activity and transmitter release in cultured neurons from hippocampus and cortex[4–7], suggesting that neurotrophins are capable of modulating synaptic function. In hippocampal slices, BDNF regulates long-term potentiation (LTP), a form of synaptic plasticity thought to be involved in learning and memory[8–10]. BDNF modulation of hippocampal LTP seems to be mediated by the ability of BDNF to enhance high-frequency transmission, through regulation of synaptic vesicle docking[9,11–13]. The simplicity and the easy accessibility of the neuromuscular junction (NMJ) make it an ideal model system to study the molecular mechanisms underlying synaptic actions of neurotrophins. Acute application of BDNF or NT-3 enhances both spontaneous and impulse-evoked transmitter release at NMJ in *Xenopus* nerve–muscle co-culture[14]. The effect of BDNF and NT3 is independent of protein synthesis[15,16]; moreover, the expression and/or secretion of some of the neurotrophins in the postsynaptic muscle cells is activity dependent, and these factors may in turn feed back on the presynaptic terminals to potentiate synaptic transmission[17,18]. BDNF exerts its effect strictly at the nerve terminals, whereas NT3 is capable of acting on terminals as well as on cell bodies[15,16]. Local perfusion experiments suggest a rapid, anterograde spreading of NT3-induced signals from soma to terminals[16]. The signals mediating the acute effect of neurotrophins are unknown.

Studies using primarily PC12 cells have identified a number of key signaling pathways activated by neurotrophins. These pathways are initiated by binding of neurotrophins to specific Trk receptor tyrosine kinases[19]. TrkA is activated by NGF, TrkB by BDNF and NT4/5, and TrkC by NT3. A general scheme based on TrkA studies is that binding of NGF to TrkA activates its tyrosine kinase activity, leading to phosphorylation on specific tyrosine residues within the intracellular domains of the receptor[20]. These phosphotyrosyl residues serve as docking sites for a number of signaling molecules, including phospholipase C-γ (PLC-γ) and Shc[21,22]. Active PLC-γ cleaves phosphatidylinositol 4,5-biphosphate (PI(4,5)P2) to generate both inositol 1,4,5-trisphosphate (IP3), which induces the release of Ca^{2+} from internal stores, and diacylglycerol, which activates protein kinase C. On the other hand, tyrosine phosphorylation of Shc triggers Shc/Grb2/Sos interaction, Ras activation and a series of phosphorylation reactions that include Raf, MEK and mitogen associated protein kinase (MAP kinase). In addition, through protein–protein interactions that involve Ras and Gab-1, Shc activates phosphoinositide 3-kinase (PI3 kinase). PI3 kinase phosphorylates the D3 position of phosphatidylinositol lipids to produce PI(3,4)P2 and PI(3,4,5)P3 (ref. 23). Taken together, binding of NGF to TrkA activates at least three major signaling pathways in PC12 cells: PLC-γ, MAP kinase and PI3 kinase.

A single neurotrophin may exert the same or distinct effects on different cell populations. Different neurotrophins, on the other hand, may act on the same type of cells and elicit similar or different biological effects. It is believed that the cellular and functional specificity is governed by coupling a specific neurotrophin and/or receptor to specific signaling pathways. Thus, signaling mechanisms

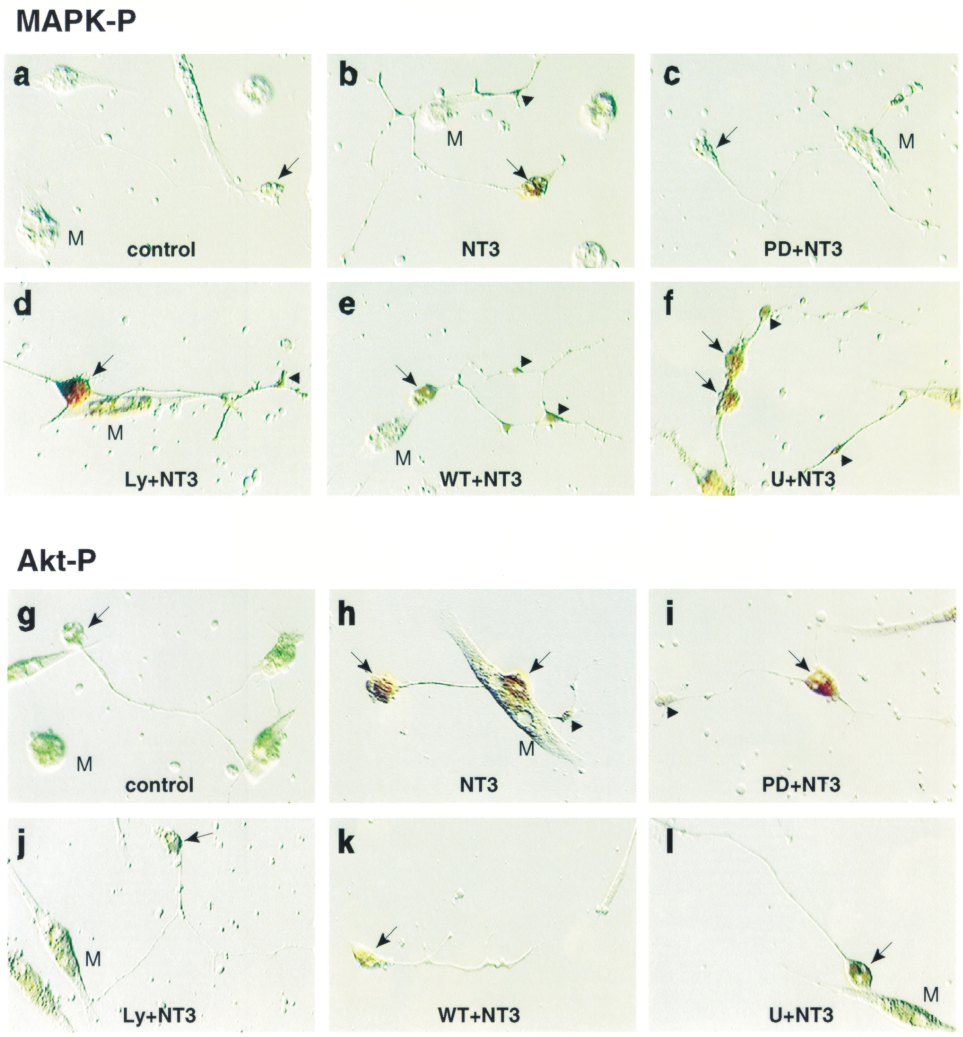


Fig. 1. Immunocytochemical detection of activation of MAP kinase and PI3 kinase pathways. *Xenopus* nerve–muscle cultures were treated with or without various inhibitors for 15–30 min, followed by application of NT-3. The cultures were fixed 15 min after NT3 application and were processed for immunocytochemistry using specific antibodies against phospho-Erk 42/44 (**a–f**) or phospho-Akt 437 (**g–l**). Arrows point to cell bodies; arrowheads indicate staining on neurites and terminals. M, muscle cell. Abbreviations in this and all other figures are as follows: WT, PI3 kinase inhibitor wortmannin; Ly, LY294002; PD, MAP kinase pathway inhibitor PD098059; U, PLC-γ inhibitor U73122.

that mediate the specific biological functions of a particular neurotrophin should be determined. For example, NGF/TrkA stimulates the survival of sympathetic neurons primarily by the PI3 kinase pathway[22]. In a study using sympathetic neurons expressing exogenous TrkB, PI3 kinase and MAP kinase pathways were shown to be required for BDNF/TrkB-mediated neuronal survival[24]. Both BDNF and NT3 attract growth cone turning of *Xenopus* motoneurons, but the BDNF effect requires Ca^{2+} influx and is dependent on $[cAMP]_i$, whereas the NT3 effect is regulated by $[cGMP]_i$ and is independent of extracellular Ca^{2+} (ref. 25). In sympathetic neurons transfected with TrkB, axonal growth is dependent on both MAP kinase and PI3 kinase pathways[24]. What are the signaling mechanisms mediating the synaptic function of neurotrophins? In the neonatal hippocampus, BDNF modulation of synaptic plasticity requires activation of MAP kinase and PI3 kinase, but not PLC-γ[26]. However, activation of the two pathways together is not sufficient to mimic the BDNF effect. At the *Xenopus* NMJ, BDNF seems to potentiate synaptic transmission by enhancing Ca^{2+} influx to presynaptic neurons, resulting in an increase in transmitter release[27]. In contrast, the acute enhancement of transmitter release by NT3 does not require Ca^{2+} influx, but instead depends on Ca^{2+} release from intracellular Ca^{2+} stores, leading to an activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)[28]. Here we examine the signaling events necessary and/or sufficient to mediate the acute modulation of synaptic transmission at the NMJ by NT3. We show that PI3 kinase and IP3 signaling pathways are both necessary and sufficient to mediate the acute effect of NT3 at the developing NMJ. Our results also implicate a general involvement of PI3 kinase in regulating basal transmitter release.

Results

NT3 activates MAP kinase, PI3 kinase and PLC-γ

NT3 signals through MAP kinase, PI3 kinase and PLC-γ pathways in certain cell populations[21,29–31]. It is unclear, however, if and how NT3 activates these signaling pathways in developing *Xenopus* spinal neurons in culture, a model system that has been extensively used

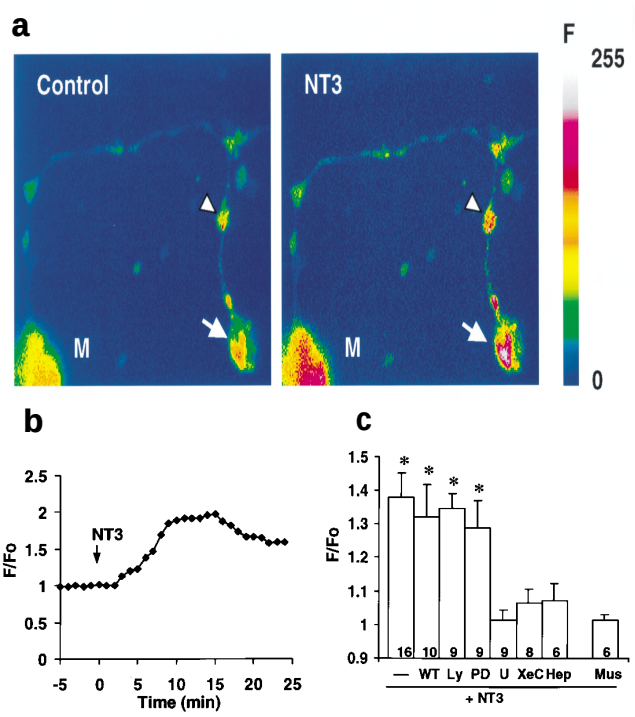


Fig. 2. NT3-induced Ca^{2+} release from intracellular stores depends on PLC-γ and IP3. The embryo injection method was used to load calcium green-dextran into spinal neurons or muscle cells. Various inhibitors were applied to the cultures for 15 min before NT3 application. (**a**) A pair of images of calcium-green-filled cells acquired 1 min before (control) and 10 min after NT3 application (NT3). Arrows, neuronal cell bodies; arrowheads, varicosities; M, muscle cell. (**b**) Time course of change in calcium green fluorescence intensity (arbitrary unit) in an isolated neuron after application of NT3. (**c**) Quantitation of fluorescence intensity using region-of-interest tool outlining neuronal cell bodies; *post-hoc* normalized to initial fluorescence (F/F_0). Heparin (Hep) was loaded into spinal neurons by embryo injection. Mus, isolated muscle cells. Error bars in this and all other figures, s.e.m. The number associated with each column represents number of cells recorded. *Groups that are significantly different from the control. One-factor ANOVA and *post-hoc* comparison; $p < 0.001$.

to study neurotrophic regulation of synaptic transmission. The limited number of cells in these cultures precluded conventional biochemical analysis. We therefore used imaging to examine NT3-induced signaling events and the specificity of various inhibitors. We examined at least 20 neurons in each condition and obtained consistent results. Activation of the MAP kinase pathway was measured by immunostaining using an antibody specific for the phosphorylated (active) form of MAP kinase (Erk1-P and Erk2-P, or MAPK-P). Application of NT3 (2–6 nM) elicited a rapid phosphorylation of MAP kinase in the spinal motoneurons (**Fig. 1**). In cultures treated with NT3 for 15 minutes, dark brown staining product accumulated not only in neuronal cell bodies (arrows) but also in the nerve terminals (arrowheads). Mild staining was also observed in some of the muscle cells (such as in **Fig. 1d**), suggesting that NT3 may also activate MAP kinase in muscle cells. PD098059 is a specific inhibitor for MEK, the enzyme that phosphorylates and activates MAP kinase[32]. Pretreatment with PD098059 (10 μM) virtually abolished the NT3-induced staining (**Fig. 1c**). In contrast, neither the PI3 kinase inhibitors LY294002 (5 μM)[33] or wortmannin (100 nM)[34], nor the PLC-γ inhibitor U73122 (5 μM)[35] had any effect on NT3-induced MAP kinase activation (**Fig. 1d–f**). The activation of PI3 kinase was detected by immunostaining using an antibody that specifically recognizes the phosphorylated Akt (Akt-P), the target of PI3 kinase. Despite background staining, application of NT3 clearly increased the staining of Akt-P (**Fig. 1g** and **h**). The increase in Akt-P staining was blocked by the PI3 kinase inhibitor LY294002 (5 μM) or wortmannin (100 nM), but not by the MEK inhibitor PD098059 (10 μM) or the PLC-γ inhibitor U73122 (5 μM; **Fig. 1i–l**). Taken together, these results demonstrate that NT3 can activate MAP kinase and PI3 kinase pathways in the developing spinal neurons.

To demonstrate the activation of PLC-γ and its downstream release of Ca^{2+} from intracellular Ca^{2+} stores, we did Ca^{2+} imaging experiments using confocal microscopy. We injected Ca^{2+} indicator dye calcium green-dextran into *Xenopus* embryos, which we then used to prepare the nerve–muscle co-culture. We applied NT3 to the culture medium, with or without various inhibitors. Because it was difficult to reliably detect cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) at the nerve terminals, we measured the intensity of calcium green fluorescence within neuronal cell bodies. The changes in $[Ca^{2+}]_i$ were quantified by normalizing the fluorescence intensity 10 min after NT3 application to the initial fluorescence intensity (F/F_0). Application of NT3 significantly increased $[Ca^{2+}]_i$ in isolated spinal neurons as well as in neurons innervating muscle cells (**Fig. 2a** and **c**). This occurred in both normal and Ca^{2+}-free media (data not shown). The change in $[Ca^{2+}]_i$ was gradual and reached a peak in approximately 10–15 min (**Fig. 2b**). Pretreatment of the cultures with either the PLC-γ inhibitor U73122 (5 μM) or IP3 receptor blocker Xestospongin C (XeC, 1 μM)[36] blocked the $[Ca^{2+}]_i$ increase (**Fig. 2c**). Introduction of another IP3 receptor inhibitor, heparin[37], into the spinal neurons by the embryo injection method also prevented the NT3 effect (**Fig. 2c**). In contrast, inhibition of the MAP kinase pathway with PD098059, or inhibition of PI3 kinase with wortmannin or LY294002 had no effect (**Fig. 2c**). In normal medium, we occasionally observed an increase in $[Ca^{2+}]_i$ in innervated muscle cells (**Fig. 2a**), possibly due to depolarization elicited by an elevated spontaneous presynaptic activity. Consistent with this observation, isolated muscle cells did not exhibit any change in $[Ca^{2+}]_i$ upon NT3 treatment (**Fig. 2c**). Thus, NT3 can induce Ca^{2+} release from intracellular stores in spinal neurons, but not muscle cells, in a PLC-γ-dependent but MAP kinase- and PI3 kinase-independent manner.

NT3 effect requires PI3 kinase and PLC-γ

Using the specific inhibitors characterized above, we determined the signaling mechanisms mediating the acute potentiation of synaptic transmission by NT3. As reported previously[14], application of NT3 to the neuromuscular synapses elicited a marked increase in the frequency of spontaneous synaptic currents (SSCs) (**Fig. 3a**). Among 15 synapses we tested, 12 showed good responses to NT3 (**Fig. 3b**). Pretreatment of the synapses with k252a (200 nM), a specific inhibitor for Trk receptor tyrosine kinases, abolished the effect of NT3 (data not shown). The increase of SSC frequency was rapid, and reached a plateau within 15–20 minutes after bath application of NT3 (**Fig. 3c**). This effect was presynaptic; the amplitude as well as rise and decay times of SSCs remained unchanged after NT3 application (data not shown)[14]. We first used wortmannin to test whether PI3 kinase is involved in the rapid, synaptic function of NT3 at NMJ. The cultures were pretreated with wortmannin (500 nM) for 15 min to inhibit PI3 kinase, and then NT3 was applied. The NT3-induced synaptic potentiation was completely blocked. Within the entire course of recording, the SSC frequency essentially remained the same (**Fig. 3c**). A lower concentration of wortmannin (100 nM), which blocks PI3 kinase but not PI4 kinase[34], also blocked the NT3 effect

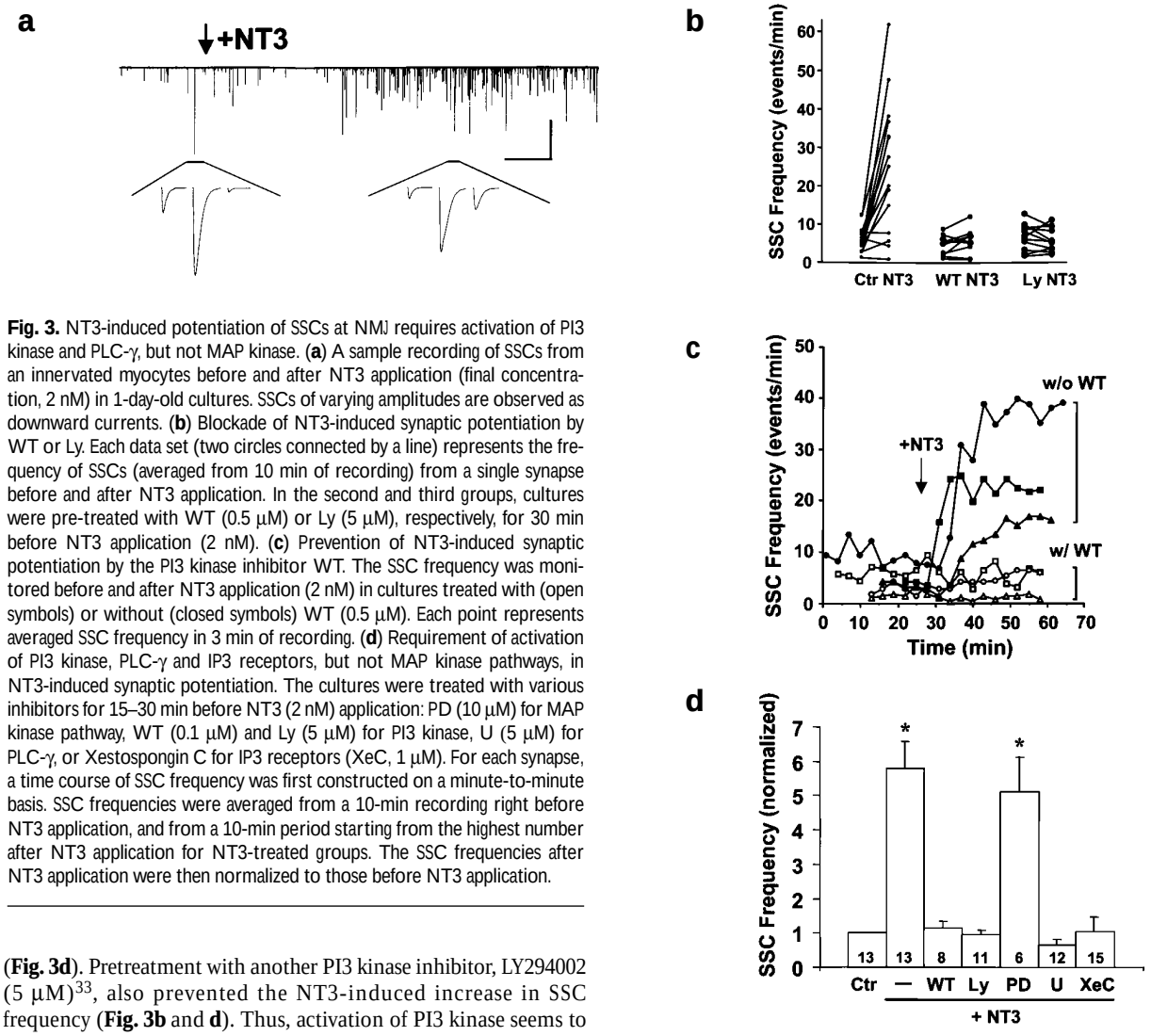


Fig. 3. NT3-induced potentiation of SSCs at NMJ requires activation of PI3 kinase and PLC-γ, but not MAP kinase. (**a**) A sample recording of SSCs from an innervated myocytes before and after NT3 application (final concentration, 2 nM) in 1-day-old cultures. SSCs of varying amplitudes are observed as downward currents. (**b**) Blockade of NT3-induced synaptic potentiation by WT or Ly. Each data set (two circles connected by a line) represents the frequency of SSCs (averaged from 10 min of recording) from a single synapse before and after NT3 application. In the second and third groups, cultures were pre-treated with WT (0.5 μM) or Ly (5 μM), respectively, for 30 min before NT3 application (2 nM). (**c**) Prevention of NT3-induced synaptic potentiation by the PI3 kinase inhibitor WT. The SSC frequency was monitored before and after NT3 application (2 nM) in cultures treated with (open symbols) or without (closed symbols) WT (0.5 μM). Each point represents averaged SSC frequency in 3 min of recording. (**d**) Requirement of activation of PI3 kinase, PLC-γ and IP3 receptors, but not MAP kinase pathways, in NT3-induced synaptic potentiation. The cultures were treated with various inhibitors for 15–30 min before NT3 (2 nM) application: PD (10 μM) for MAP kinase pathway, WT (0.1 μM) and Ly (5 μM) for PI3 kinase, U (5 μM) for PLC-γ, or Xestospongin C for IP3 receptors (XeC, 1 μM). For each synapse, a time course of SSC frequency was first constructed on a minute-to-minute basis. SSC frequencies were averaged from a 10-min recording right before NT3 application, and from a 10-min period starting from the highest number after NT3 application for NT3-treated groups. The SSC frequencies after NT3 application were then normalized to those before NT3 application.

(**Fig. 3d**). Pretreatment with another PI3 kinase inhibitor, LY294002 (5 μM)[33], also prevented the NT3-induced increase in SSC frequency (**Fig. 3b** and **d**). Thus, activation of PI3 kinase seems to be required for the acute effect of NT3.

We used PD098059 to examine the involvement of MAP kinase in the acute effect of NT3 at the neuromuscular synapses. Although PD098059 (10 μM) blocked MAP kinase activation (**Fig. 1c**), it did not hamper the NT3-induced increase in SSC frequency (**Fig. 3d**), suggesting that MAP kinase is not involved in the acute effect of NT3. In contrast, inhibition of PLC-γ by U73122 (5 μM) completely prevented the NT3-induced increase in SSC frequency (**Fig. 3d**). Pretreatment of the nerve–muscle co-culture with U73334 (5 μM), an inactive analog of U73122, did not hinder the potentiating effect of NT3 (the ratio of SSC frequencies after and before NT3 application, 6.31). Consistent with this result, inhibition of IP3 receptor by XeC (1 μM) also abolished the NT3 effect (**Fig. 3d**). Thus, the synaptic potentiation induced by NT3 requires PI3 kinase and PLC-γ, two of the three major signaling pathways for neurotrophins.

NT3 effect requires continuous activation of PI3 kinase

PI3 kinase has two subunits, p110 and p85; fusion of the p110 catalytic subunit with the inter-SH2 fragment of p85 subunit results in a constitutively active form of the kinase (PI3K*), whereas the same chimeric molecule can serve as a dominant negative form (PI3K#) if the kinase domain is mutated[38]. To further characterize the involvement of PI3 kinase in NT3-induced synaptic potentiation, we targeted PI3K# into motoneurons by embryo injection. Because the acute effect of NT3 on synaptic transmission is independent of Ca^{2+} influx[28], we determined whether the acute effect of NT3 can be blocked by PI3K# in Ca^{2+}-free medium. PI3K# mRNA was injected with green fluorescence protein (GFP) mRNA into one of the blastomeres of *Xenopus* embryos at two-cell stage. Nerve–muscle cultures were prepared from the injected embryos one day later. Introduction of PI3K# did not seem to affect the development and morphology of the *Xenopus* embryos at the time of cell culture (data not shown). Western blot analysis using an antibody against the Myc tag on the PI3K# detected a substantial amount of intact PI3K# (145 kDa) in 1-day (1-d) embryos (**Fig. 4b**), indicating that the chimeric PI3K# protein was expressed. The spinal neuron and the myocyte one-day-old cultures also exhibited normal morphology (**Fig. 4a**). GFP fluorescence, which reflects the distribution of injected mRNA[17], served as an indicator of PI3K# expression. In cultures derived from injected embryos, substantial number of neurons and myocytes were GFP positive (N+ and M+, **Fig. 4a**).

Overexpression of PI3K# had no effect on the basal level of spontaneous transmitter release (**Fig. 4c** and **d**). The frequency of SSC recorded from PI3K#+ and PI3K#– synapses were very similar (2.42 ± 0.59 events/min versus 1.96 ± 0.49 events/min). However, NT3 could no longer elicit a potentiation of synaptic transmission

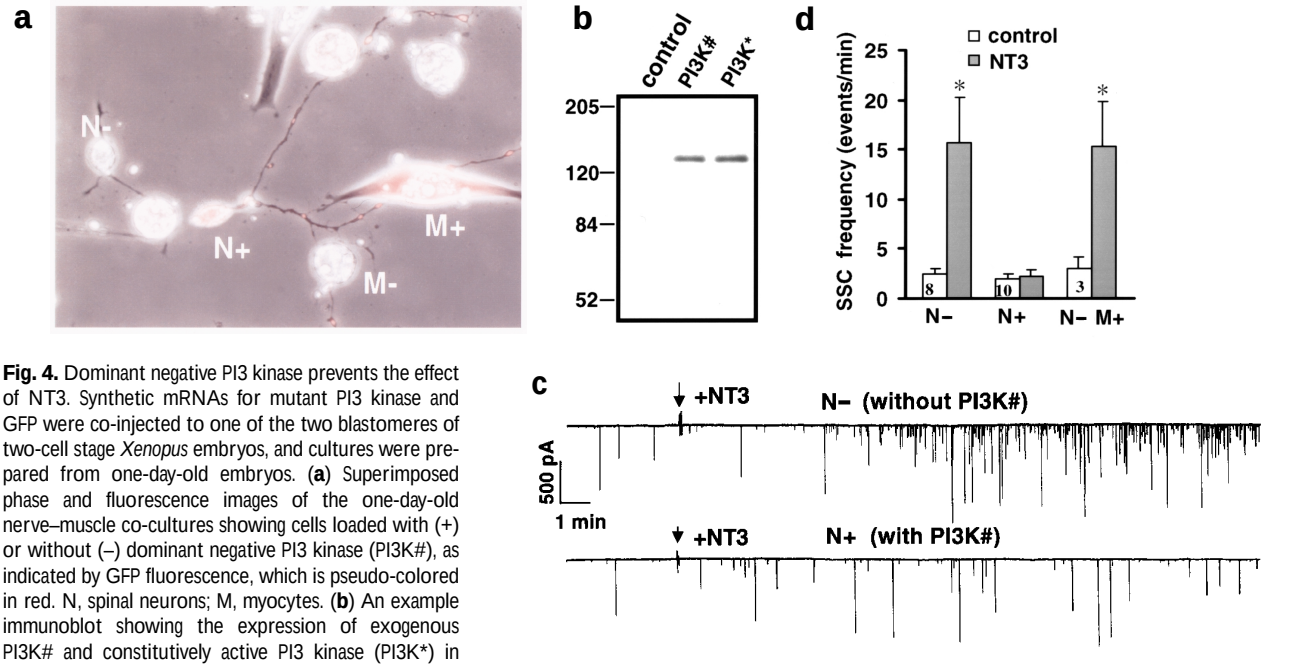


Fig. 4. Dominant negative PI3 kinase prevents the effect of NT3. Synthetic mRNAs for mutant PI3 kinase and GFP were co-injected to one of the two blastomeres of two-cell stage *Xenopus* embryos, and cultures were prepared from one-day-old embryos. (**a**) Superimposed phase and fluorescence images of the one-day-old nerve–muscle co-cultures showing cells loaded with (+) or without (–) dominant negative PI3 kinase (PI3K#), as indicated by GFP fluorescence, which is pseudo-colored in red. N, spinal neurons; M, myocytes. (**b**) An example immunoblot showing the expression of exogenous PI3K# and constitutively active PI3 kinase (PI3K*) in stage 24 embryos. Myc-tagged PI3K# or PI3K* mRNA (final concentration, 10 ng/ml) was injected into *Xenopus* embryos at the 2-cell stage, and the levels of PI3K# or PI3K* protein in the stage-24 embryos were measured by western blot using a monoclonal antibody against Myc. Molecular weight markers are indicated on the side of the gel (in kDa). A single band of 145 kDa appears in embryos injected with PI3K# mRNA or PI3K* mRNA, but not in control, uninjected embryos. (**c**) A pair of recordings showing that loading of PI3K# into presynaptic neurons N+ prevented the NT3-induced increase in SSC frequency, whereas NT3 still enhanced transmitter release in an N– neuron in the same culture. (**d**) Summary of the effect of PI3K# expressed in either presynaptic spinal neurons or postsynaptic myocytes. The number of synapses recorded are indicated in each pair of columns. PI3K# is effective in inhibiting NT3 effect when expressed in presynaptic neurons (N+) but not postsynaptic (M+) myocytes ($p < 0.001$, Student's *t*-test).

in synapses made by PI3K#+ neurons (N+ in **Fig. 4c** and **4d**). In contrast, application of NT3 to PI3K#– synapses in the same dish elicited an increase in SSC frequency similar to the increase observed in regular cultures (N– in **Fig. 4c** and **4d**). Furthermore, when PI3K# was expressed in the postsynaptic myocytes (M+), NT3 was still capable of eliciting a significant increase in SSC frequency (N–M+ in **Fig. 4d**). Taken together, these data strongly suggest that presynaptic, but not postsynaptic, activation of PI3 kinase is necessary for NT3-induced synaptic potentiation.

The acute potentiation of synaptic transmission at NMJ depends on continued presence of NT3, because withdrawal of NT3 reduces the synaptic efficacy to control level[14]. To examine whether the NT3-induced synaptic potentiation requires a continuous activation of PI3 kinase, we applied wortmannin (0.5–1 μM) or LY294002 (5 μM) after synaptic transmission was elevated by NT3. Within approximately 10 minutes after the addition of NT3 into the culture dishes, the increase in SSC frequency reached a peak (**Fig. 5a**). Application of wortmannin at the peak gradually suppressed the SSC frequency increased by NT3. Quantitative analysis indicated that LY294002 virtually reversed the NT3 effect (**Fig. 5b**). Similar results were observed when wortmannin was used to inhibit PI3 kinase (**Fig. 5b**). These data suggest that NT3 modulation of synaptic transmission requires continuous activation of PI3 kinase.

Sufficiency of co-activation of PI3 kinase and IP3 receptors

To address whether activation of PI3 kinase is sufficient to enhance synaptic transmission, we used a constitutively active form of PI3 kinase (PI3K*). Expression of the PI3K*-containing construct in HEK cells resulted in a marked increase in PI3 kinase activity (data not shown). Messenger RNA for PI3K* was obtained by *in vitro* transcription and was targeted to the motoneurons with embryo injection. A substantial amount of intact PI3K* (145 kDa) was also detected in one-day-old embryos by western blot (**Fig. 4b**). SSC frequencies recorded from synapses made by PI3K*+ neurons were very similar to frequencies recorded from PI3K*– neurons (**Fig. 6a**). Quantitative analysis indicated that PI3K*+ and PI3K*– neurons exhibited almost the same levels of spontaneous transmitter release (4.3 ± 0.9 versus 5.9 ± 0.8 events per minute, respectively). Furthermore, application of NT3 still elicited the same magnitude of increase in SSC frequency in PI3K*+ synapses (29.9 ± 5.3 events per minute) as in PI3K*– synapses (27.2 ± 5.4 events per minute; **Fig. 6a**). Taken together, these results demonstrate that a constitutive activation of PI3 kinase alone in motoneurons is not sufficient to elicit synaptic potentiation.

Because the acute effect of NT3 can be blocked by inhibitors of either PI3 kinase or PLC-γ pathway (**Fig. 3**), it is possible that NT3-induced synaptic potentiation requires a concomitant activation of PI3 kinase and PLC-γ. We therefore tested whether an increase in intracellular IP3, a major downstream product of PLC-γ, together with PI3 kinase activation, is sufficient to enhance synaptic transmission. Motoneurons were loaded with caged IP3 through embryo injection. In the first series of experiments, caged IP3 was injected alone into one of the blastomeres in the *Xenopus* embryos at two-cell stage using rhodamine as an indicator. A brief ultraviolet (UV) light exposure to the cells loaded with caged IP3 rapidly and reliably releases caged IP3 (photo-uncaging), leading to an increase the intracellular Ca^{2+} concentration[39]. However, flashing UV light to cells (1 second, λ = 340–400 nm) either with or without caged

IP3 (N+ or N– cells) did not elicit any changes in SSC frequency (**Fig. 6b**, left), suggesting that an increase in IP3 alone is not sufficient to enhance transmission. Second, we loaded PI3K* mRNA into the spinal neurons through embryo injection using GFP as an indicator. Exposure of UV light to spinal neurons expressing PI3K* had no effect on synaptic activity at the NMJ (data not shown). Finally, we loaded both caged IP3 and PI3K* mRNA into the same spinal neurons. Photo-uncaging of IP3 in motoneurons containing both caged IP3 and PI3K* resulted in a marked increase in SSC frequency (N+ in **Fig. 6b**, right). The magnitude of synaptic potentiation was almost identical to the magnitude elicited by NT3 (compare **Fig. 6b** with **3d**). In contrast, no change in the magnitude of synaptic activity was observed in NMJ made by unloaded neurons (N–, **Fig. 6b**, right) in the same cultures when exposed to UV light. Further, UV flash had no effects on SSC frequency in synapses in which PI3K* and caged IP3 were loaded in postsynaptic muscle cells (N– M+, **Fig. 6b,** right).

To determine whether synaptic potentiation elicited by photolysis of caged IP3 in PI3K*+ neurons is truly due to the concomitant activation of PI3 kinase and IP3 receptors, we tested the effects of various inhibitors. Only neurons loaded with both caged IP3 and PI3K* were used in these experiments. Pretreatment of the cultures with either wortmannin (100 nM) or LY294002 (5 μM) blocked the increase in SSC frequency elicited by combined Ca^{2+} mobilization and activated PI3 kinase (**Fig. 6c**). Similarly, application of XeC (1 μM) or loading of heparin (estimated intracellular concentration, 100 μg/ml) by embryo injection prevented the synaptic potentiation evoked by photo-uncaging of IP3 (**Fig. 6c**). However, release of IP3 by photo-uncaging still enhanced transmission in the presence of U73122 (5 μM), which inhibits PLC-γ but not its downstream IP3 receptors (**Fig. 6c**). Taken together, these results indicate that simultaneous activation of PI3 kinase and release of IP3 in the presynaptic neurons is sufficient to potentiate synaptic transmission at the NMJ, a mechanism very likely to mediate the acute effect of NT3.

Physiological role of PI3 kinase and IP3 receptors

The requirement for both PI3 kinase and IP3 receptors in the NT3-induced increase in transmitter release predicts the involvement of these molecules in functional synaptic transmission, as reflected by the impulse-evoked synaptic currents (ESCs). We therefore applied supra-threshold stimuli to the neuronal soma to fire action potentials, and recorded ESCs from neuromuscular synapses. Consistent with a previous report[14], application of NT3 significantly increased the amplitude of ESCs (**Fig. 7**). The effect of NT3 was completely blocked in the spinal neurons overexpressing the dominant negative form of PI3 kinase (PI3K#, **Fig. 7b**). However, overexpression of a constitutively active form of PI3 kinase (PI3K*) did not further increase the ESC amplitude (**Fig. 7b**). Moreover, blockade of IP3 receptors by XeC (1 μM) also attenuated the NT3-induced increase in ESC amplitude (**Fig. 7b**). Thus, activation of both PI3 kinase and IP3 receptors is required for NT3 modulation of functional synaptic transmission.

To investigate whether PI3 kinase and the IP3 receptor mediate the NT3 effect at the developing neuromuscular synapses *in vivo*, we recorded miniature endplate potentials (MEPPs) in the tail muscles of *Xenopus* tadpoles (stage 36–40). To avoid muscle contraction, the Na^+ channel blocker tetrodotoxin (3 μM) was included in Ringer's solution. Insertion of the intracellular recording electrode into the muscle fibers near the NMJ detected MEPPs (**Fig. 8a**), and the shape of the MEPPs was very similar to those previously reported[40]. Although the frequencies of MEPPs varied depending on the individual muscle cells recorded, application of NT3 (6 nM) reliably increased the MEPP frequency (**Fig. 8a** and **b**). NT3 also elicited a slight hyperpolarization of the postsynaptic muscle membrane under the current-clamp conditions, resulting in a reduction of the width of MEPP waveforms (**Fig. 8a**). We normalized the MEPP frequencies after NT3 application to those before NT3 application. Pretreatment with the PI3 kinase inhibitor LY294002 (5 μM) completely prevented the rise of MEPP frequency elicited by NT3 (**Fig. 8b**). Inhibition of the IP3 receptors by XeC (1 μM) also prohibited the NT3-induced increase in spontaneous synaptic transmission (**Fig. 8b**). These results suggest that NT3 is involved in modulating synaptic transmission at the developing neuromuscular synapses *in vivo*, and that PI3 kinase and IP3 receptor are required in such modulation.

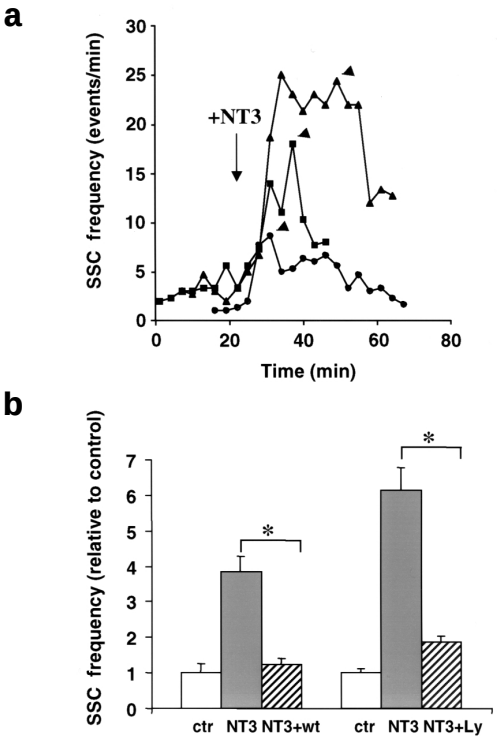


Fig. 5. Continuous activation of PI3 kinase is required for NT3-induced synaptic potentiation. (**a**) Examples showing the time courses for PI3 kinase inhibitor WT reversal of the NT3 effect. NT3 was first applied (arrow) to the culture medium to elicit an increase in the frequency of SSCs. After the SSC frequency reached the peak, WT (arrowhead) was applied, and synaptic currents were continuously monitored. (**b**) Summary of the reversal effect of WT and Ly. SSC frequencies are calculated in the same way as for Fig. 3d. $n = 5$ for both WT and Ly groups. $*p < 0.01$, Student's *t*-test.

Discussion

Gene expression in the *Xenopus* neuromuscular synapse can be manipulated with simplicity and ease. Thus, we were able to address signaling mechanisms, at the molecular level, that mediate synaptic actions of neurotrophins. We have demonstrated that the acute potentiation of transmitter release by NT3 at the neuromuscular synapses is mediated, not by Ca^{2+} influx from extracellular sources, but by Ca^{2+} released from intracellular stores through IP3 receptors and activation of CaMKII[28]. In this study, we systematically investigated signaling pathways involved in NT3-induced synaptic potentiation. We showed that the effect of NT3 requires activation

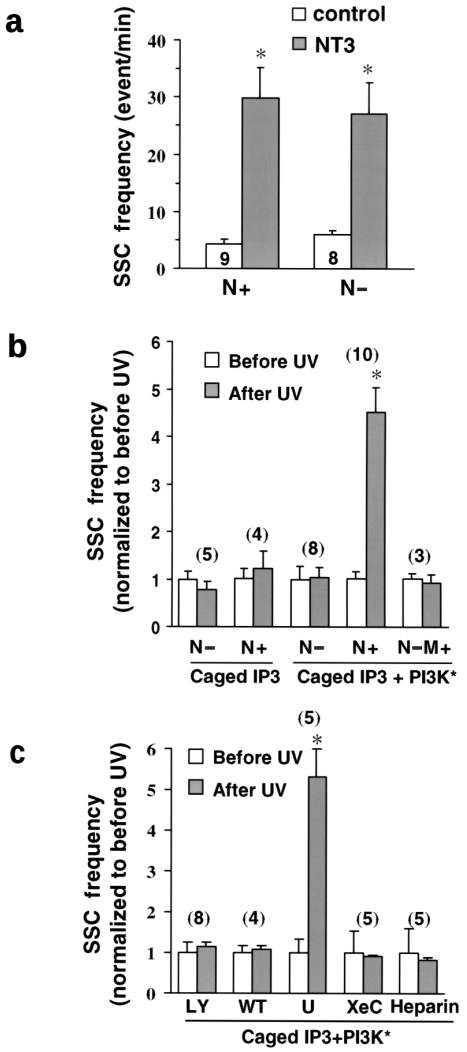


Fig. 6. Concomitant activation of PI3 kinase and IP3 receptors enhances transmitter release. (**a**) Activation of PI3 kinase alone is not sufficient to enhance spontaneous synaptic activity. PI3K* was expressed in motoneurons or myocytes by embryo injection techniques. SSCs before and after NT3 application were monitored in both N+ and N– neurons. Presynaptic expression of PI3K* did not affect basal spontaneous synaptic activity, and application of NT3 still elicited a marked increase in SSC frequency. (**b**) Simultaneous activation of PI3 kinase and IP3 receptors is sufficient to potentiate spontaneous synaptic activity. Neurons were loaded with caged IP3, PI3K* or both, through embryo injection. SSCs were recorded from N+ and N– synapses. After approximately 10–30 min of control recording, the cells were exposed to UV light for 1 s. For each synapse, SSC frequency, calculated from a 10-min period starting from the highest number after UV flash (UV), was normalized to that from a 10-min recording right before UV flash. The averaged data are presented. (**c**) Enhancement of transmitter release elicited by co-activation of PI3 kinase and IP3 receptors is blocked by LY294002, wortmannin, Xestospongin C and heparin, but not by U73122. Heparin was loaded into spinal neurons by embryo injection. Cultures were treated with various inhibitors before UV flash. Experiments were done in the same way as above except only neurons loaded with both caged IP3 and PI3K* were used.

of PI3 kinase and PLC-γ pathways, but not MAP kinase pathway. Further, we demonstrated, using photo-uncaging, that simultaneous activation of PI3 kinase and IP3 receptors is not only necessary to mediate the effect of NT3, but is also sufficient to mimic it. Also, NT3 modulation of evoked transmission and transmitter release at the NMJ require PI3 kinase and IP3 receptors in the developing tadpole *in vivo*. Thus, NT3-induced synaptic potentiation uses PI3 kinase and PLC-γ, two of the three major signaling pathways for neurotrophins. In theory, any factors (extracellular and/or intracellular) that can influence these pathways should be able to regulate neurotransmitter release at the NMJ. Thus, our findings not only help explain the mechanisms of acute, synaptic actions of neurotrophins, but also help explain the signaling mechanisms involved in the regulation of neurotransmitter release.

A fundamental issue in neurobiology is how signaling molecules achieve cellular and functional specificity in the complex nervous system. Neurotrophins seem to achieve specificity not by tissue-specific expression of specific Trk receptors, but by the combinatorial use of multiple signaling pathways. Here we consider three situations of such use. First, the same factor acts on the same neuronal population but elicits totally different effects at different time scales. For example, NT3 chronically promotes the survival of TrkC-expressing spinal motoneurons[1], but acutely enhances transmitter release from them[14]. We show that NT3-induced synaptic potentiation at NMJ requires the activation of PI3 kinase and IP3 receptors, but not MAP kinase. The survival of motoneurons is mediated essentially by the PI3 kinase pathway alone[41]. Second, different cell populations may have very different responses to the same neurotrophin. In *Xenopus* motoneurons, NT3 activates all three signaling pathways, two of which, the PI3 kinase and PLC-γ/IP3 receptor pathways, are both necessary and sufficient to mediate the NT3-induced potentiation of transmitter release. In mammalian hippocampal slices, TrkC receptors are highly expressed in the neonatal hippocampus[42,43], but application of NT3 does not activate MAP kinase or PI3 kinase[26]. NT3 has no effect on basal transmitter release, but regulates short-term synaptic plasticity in dentate granule cells[44]. This is not due to species differences, because human recombinant NT3 has been used to elicit diverse biological effects in both mouse and *Xenopus*. Thus, NT3 may use different sets of signaling pathways in hippocampal neurons and motoneurons to elicit different biological functions. Third, different neurotrophins elicit the same biological effects through distinct signaling mechanisms. Both BDNF and NT3 attract growth cone turning in the developing motoneurons. However, the BDNF effect requires Ca^{2+} influx and is dependent on $[cAMP]_i$, whereas the NT3 effect requires $[cGMP]_i$ and is independent of extracellular Ca^{2+} (ref. 25). We show here that NT3 enhances transmitter release at the NMJ by activating PI3 kinase and IP3 receptors. BDNF also potentiates transmitter release at these synapses, but this effect is dependent on Ca^{2+} influx, rather than IP3-induced increase in $[Ca^{2+}]_i$ (refs. 27, 28). Although we cannot rule out the possibility that NT3 and BDNF activate TrkB in qualitatively different manners, our study strongly supports the notion that BDNF and NT3 use distinct signaling mechanisms to regulate transmitter release at NMJ.

Our results also implicate the PI3 kinase in basal transmitter release. The regulation of neuronal survival and apoptosis is thought to be the major function of PI3 kinase in the nervous system[45], but very little is known about the involvement of PI3 kinase in synaptic function. In the hippocampus, inhibition of PI3 kinase either by pharmacological and genetic approaches has been shown to affect LTP[46]. We showed previously that PI3 kinase is required for BDNF modulation of synaptic fatigue, a form of synaptic plasticity elicited by high-frequency repetitive stimulation[26]. However, these studies did not address whether PI3 kinase regulates the basal transmitter release mechanism. Here we show that inhibition of PI3 kinase prevented NT3-induced potentiation of spontaneous synaptic activity. An activation of PI3 kinase using PI3K*, together with an increase

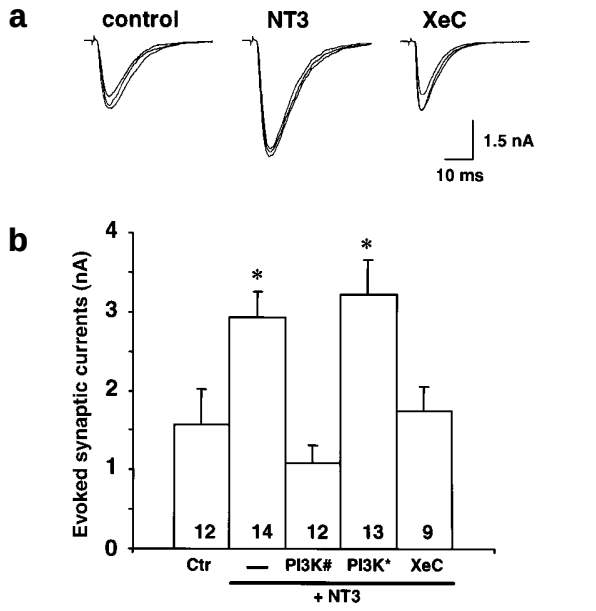


Fig. 7. NT3-induced enhancement of evoked synaptic transmission is blocked by inhibition of PI3 kinase or IP3 receptors. The presynaptic neuron was stimulated at the soma at low frequency (0.05 Hz), and evoked synaptic currents (ESCs) were recorded in the voltage-clamped condition (V_h = –70 mV). (**a**) Examples of ESCs recorded from synapses in control, after NT3 application, or XeC + NT3. (**b**) Effects of PI3#, PI3K* and XeC on NT3-induced enhancement of ESCs. PI3# or PI3K* was loaded into presynaptic neurons through embryo injection. XeC was applied 30 min before NT3 application.

in IP3 through photo-uncaging, elicited a significant enhancement of transmitter release in the absence of NT3. However, either PI3K* or uncaging of IP3 alone was not enough to enhance transmitter release. Taken together, these experiments suggest that activation of PI3 kinase may be a critical step in transmitter release or vesicle fusion mechanism, as long as $[Ca^{2+}]_i$ is elevated (either by Ca^{2+} influx or Ca^{2+} release from intracellular stores). These results provide physiological evidence for a link between PI3 kinase and basal transmitter release.

Future studies should explore how PI3 kinase facilitates vesicle fusion. PI3 kinase phosphorylates phosphatidylinositol on the 3 position, generating lipid products including PI(3,4)P2, and PI(3,4,5)P3. The downstream targets of PI3 kinase usually contain a pleckstrin-homology (PH) domain that binds to these lipids[23]. Synaptotagmin, a protein that is important in regulating synaptic vesicle fusion, may be a potential downstream target of PI3 kinase[47]. The C2B domain of synaptotagmin binds with high affinity to PI(3,4,5)P3 at resting levels of Ca^{2+}. An increase in $[Ca^{2+}]_i$, either through Ca^{2+} influx during evoked synaptic transmission or release of Ca^{2+} from intracellular stores, could switch the specificity of binding from PI(3,4,5)P3 to PI(4,5)P2 (ref. 47). It is conceivable that synaptotagmin bound to PI(3,4,5)P3 in synaptic vesicles could sense a rise in cytosolic Ca^{2+}, leading to vesicle fusion. Sufficient amounts of PI(3,4,5)P3, which are generated during NT3 application, may be required for synaptic vesicles to be 'primed' for the fusion event.

METHODS

Constructs of PI3 kinase mutants and embryo injection. Fusion of the Myc tagged p110 subunit, with the inter SH2 domain of p85 subunit of PI3 kinase, results in a constitutively active form of PI3 kinase (PI3K*) that is independent of lipid activation. However, this chimeric fusion molecule can be used as a dominant negative form of PI3 kinase (PI3K#) when it is catalytically inactive[38]. The cDNAs of PI3K# and PI3K* were digested and subcloned into an expression vector pcDNA3(–) (Invitrogen, Carlsbad, California), which contained a T7 promoter for *in vitro* transcription of sense mRNA. The capped mRNAs of PI3 kinase mutants and GFP were transcribed *in vitro* with a T7 RNA polymerase, using the mMESSAGE mMACHINE kit (Ambion, Austin, Texas)[17]. *Xenopus* egg laying was induced by injecting a female *Xenopus* with human chorionic gonadotropin, and eggs were fertilized artificially with sperm derived from a male testis. PI3 kinase mutant mRNAs were mixed with GFP mRNA (1:1) and a total of 10 ng of mRNAs was injected into one of the cells at two-cell stage embryos using the Picospitzer pressure ejector (Parker Hannifin, Fairfield, New Jersey). The same embryo injection method was used to load calcium green-dextran or the IP3 receptor inhibitor, heparin, into spinal neurons. At stage 20–22, the injected embryos were used to prepare nerve–muscle co-cultures as described below. Unless indicated otherwise, all chemicals were from Sigma (St. Louis, Missouri) and all media and sera were from Gibco BRL (Rockville, Maryland).

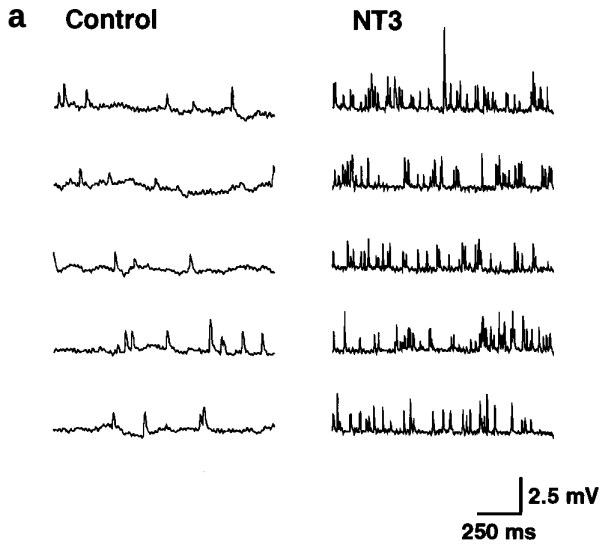
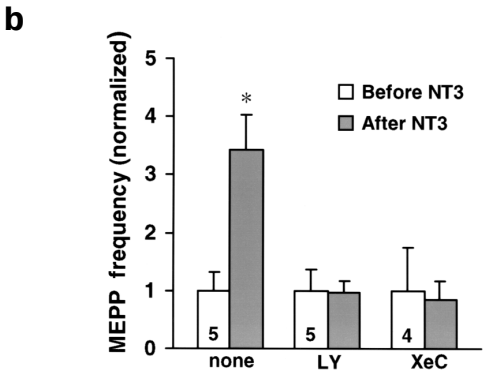


Fig. 8. NT3 potentiates spontaneous transmitter release at the NMJ in *Xenopus* tadpoles *in vivo*. (**a**) Examples of MEPPs recorded from a single tadpole muscle fiber before (left) and after (right) bath application of NT3. (**b**) Effects of Ly and XeC on NT3-induced enhancement of MEPPs *in vivo*. In each condition, MEPP frequency in each cell recorded was normalized to the mean frequency before NT3 application.



Western blotting. *Xenopus* embryos at stage 20 or 40 were quickly homogenized in extraction buffer (100 μM NaCl, 50 μM Tris-HCl, pH 7.5, 1% NP-40, 2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μM Na_3VO_4) followed by sonication. The insoluble yolk proteins were discarded after centrifugation. The supernatants were transferred to a fresh tube containing 300 μl Freon (1,1,2-trichlorotrifluoroethane), vortexed for 1 min, and incubated on ice for 5 min. The proteins extracted from a single embryo were separated by SDS-polyacrylamide electrophoresis, and blotted onto Immobilon-P (Millipore, Bedford, Massachusetts). The blots were probed with monoclonal anti-Myc antibody (1:500), followed by a secondary antibody conjugated with HRP, and PI3 kinase was detected by the chemiluminescence method (Pierce, Rockford, Illinois).

Culture preparation. *Xenopus* neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos (stage 20 to 22) were dissociated in Ca^{2+}-Mg^{2+}-free saline supplemented with EDTA (58.2 μM NaCl, 0.7 μM KCl, 0.3 μM EDTA, pH 7.4) for 15–20 min. The cells were plated on clean glass coverslips, and grown for one day at room temperature (20–22°C). The culture medium consisted of 50% Leibovitz (Gibco BRL, Rockville, Maryland) L-15 medium, 1% fetal calf serum and 49% Ringer's solution (115 μM NaCl, 2 μM $CaCl_2$, 2.5 μM KCl, 10 μM HEPES, pH 7.6). Various inhibitors and NT3 were applied directly to culture media at time of recording. For cultures prepared from embryos injected with PI3K# and GFP mRNAs, fluorescence images of the cultured cells were acquired by a MicroMax (Roper Scientific, Trenton, New Jersey) 1300 cool CCD camera mounted on a Nikon (Melville, New York) microscope, and assigned to a pseudo color (red). The phase and fluorescence images were superimposed using the IPLab (Scanalytics, Fairfax, Virginia) software. Previous experiments had indicated that the GFP-positive cells faithfully reflect the cells containing exogenous genes[17].

Immunocytochemistry. *Xenopus* nerve–muscle cultures were treated with or without various inhibitors for 15–30 min at room temperature before NT-3 application. The cultures were fixed 15 min after NT3 application with 3% paraformaldehyde and 0.25 glutaraldehyde (EM Science, Gibbstown, New Jersey) for 15 min at room temperature, and washed 3 times with phosphate buffer saline (PBS). Fixed cells were made permeable with 0.25% Triton X-100 for 10 min. After an incubation in blocking solution (50% normal goat serum in PBS) for 3 h, cells were incubated with primary antibodies against phospho-Akt 437 1:100 (polyclonal, Promega, Madison, Wisconsin) or phospho-Erk 42/44 1:250 (polyclonal, Cell Signaling, Beverly, Massachusetts) at 4°C overnight. Antibodies were diluted in 3% BSA/PBS. The specific labeling of Akt-P or MAPK-P was visualized using an ABC kit (Vector, Burlingame, California) following the manufacturer's instructions. Cells were dehydrolyzed and mounted onto glass slides with a mounting solution (Fisher, Neward, Delaware). The images of immunocytochemistry were captured by a CCD camera (Optronics Engineering, Goleta, California) and exported to a desktop computer. At least 20 neurons from several different batches of cultures were examined for each condition, and consistent results were obtained.

Ca^{2+} imaging. We injected embryos with calcium green-dextran (final concentration, 50 μM; Molecular Probes, Eugene, Oregon), and processed them for nerve–muscle co-culture as described above. One-day-old cultures were examined by confocal microscopy in a Noran Odyssey II confocal microscope with a fluorescein filter set. Both calcium-green-filled neurons and muscle cells were observed in many fields, along with many unfilled cells. We applied various inhibitors to the cultures for 15 min before NT3 application. For a single calcium-green-filled cell, fluorescence images were acquired twice: 1 min before, and 10 min after NT3 application. Fluorescence intensity was measured using a region-of-interest tool outlining the cell body, corrected for photo bleaching, and *post-hoc* normalized to initial fluorescence (F/F_0).

Whole-cell recording in culture. We recorded synaptic currents from innervated muscle cells by whole-cell recording methods[48], at room temperature in culture medium. The solution inside the whole-cell recording pipette contained 150 μM KCl, 1 μM NaCl, 1 μM MgCl2 and 10 μM HEPES buffer (pH 7.2). The membrane potentials of the muscle cells were generally in the range of –55 to –75 mV and were voltage clamped at –70 mV after measuring the membrane potentials. For experiments done in the absence of external Ca^{2+}, the culture medium was replaced with a Ca^{2+}-free extracellular solution containing 115 μM NaCl, 2 μM $MgCl_2$, 10 μM HEPES, 3 μM EGTA and 0.1% BSA. All data were collected by an Axonpatch 200B patch clamp amplifier (Axon Instruments, Foster City, California), with a current signal filter at 3 kHz. The data were stored on a videotape recorder for later playback on a Tektronix TDS 420 storage oscilloscope (Beaverton, Oregon) and a Gould EasyGraf 240 chart recorder (Valley Veiw, Ohio), or analysis by the Dagan SCAN program (University of Strathclyde, Glasgow, UK). To quantitatively measure the changes in neurotransmitter release, a time course of SSC frequency was first constructed on minute-to-minute basis. The SSC frequencies in a 10-min period right before NT3 (or UV light) application were averaged as control. The changes in SSC frequency were measured by averaging a 10-min period recording starting from the highest number after NT3 (UV light) application. To elicit ESCs, square current pulses (0.5 ms, 0.5 to 5 V) were applied through a patch electrode filled with Ringer's solution at neuronal soma under loose seal conditions.

Photolysis of caged IP3. Caged IP3 (Molecular Probes) was loaded into spinal neurons by embryo injection at two-cell stage (final intracellular concentration of caged IP3 was approximately 15 μM) either alone or together with PI3K*. Photolysis of caged IP3 to liberate free intracellular IP3 was achieved using flashes of UV light (340–400 nm) derived from a mercury arc lamp[39]. Synaptic currents before and after a UV flash were measured and compared. A UV flash of 1 s was sufficient to induce an increase in SSC frequency in neurons loaded with both caged IP3 and PI3*. Cells loaded with caged IP3 were identified by rhodamine (alone) or GFP fluorescence (together with PI3K*) after electrophysiological recordings.

Electrophysiological recording of *Xenopus* tadpoles. We dissected *Xenopus* tadpoles (stage 36–40) in Ringer's solution, and removed the skin, head and viscera. The skinned tails were transferred to sylgard plates, laid on their side and pinned. Included in the Ringer's solution was $CaCl_2$ (10 μM) to improve membrane resealing, and the Na^+ channel blocker, tetrodotoxin (3 μM), to avoid muscle contraction[40]. The intracellular recording electrode was filled with 3 M potassium acetate (pipette resistance, 50–55 MΩ). MEPPs were recorded at room temperature by inserting the electrode into a muscle fiber close to the motor nerve terminals. Data were collected by Axonclamp 2B amplifier (filtered at 3 kHz; Axon Instruments), displayed on a Tektronix TDS 420 storage oscilloscope (Beaverton, Oregon), or analysis by a pClamp 8 software (Axon Instruments). The tadpoles were treated with or without various inhibitors for at least 30 min before bath application of NT-3 (6 nM). MEPP frequencies are calculated by averaging from a 5-min recording right before NT3 application for controls, and from a 10-min period starting from the highest number after NT3 application for NT3-treated groups. The ratios of MEPP frequencies after and before NT3 application were used to measure the changes in the efficacy of synaptic transmission.

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