Neurotrophin 3 induces structural and functional modification of synapses through distinct molecular mechanisms

Hyun-Soo Je,1,3 Feng Yang,1 Jiangzheng Zhou,1 and Bai Lu1,2

1Section on Neural Development and Plasticity, National Institute of Child Health and Human Development, and 2Gene, Cognition and Psychosis Program, National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892
3Genetics Graduate Program, George Washington University, Washington, DC 20052

The mechanisms by which neurotrophins elicit long-term structural and functional changes of synapses are not known. We report the mechanistic separation of functional and structural synaptic regulation by neurotrophin 3 (NT-3), using the neuromuscular synapse as a model. Inhibition of cAMP response element (CRE)–binding protein (CREB)–mediated transcription blocks the enhancement of transmitter release elicited by NT-3, without affecting the synaptic varicosity of the presynaptic terminals. Further analysis indicates that CREB is activated through Ca2+/calmodulin-dependent kinase IV (CaMKIV) pathway, rather than the mitogen-activated protein kinase (MAPK) or cAMP pathway. In contrast, inhibition of MAPK prevents the NT-3–induced structural, but not functional, changes. Genetic and imaging experiments indicate that the small GTPase Rap1, but not Ras, acts upstream of MAPK activation by NT-3. Thus, NT-3 initiates parallel structural and functional modifications of synapses through the Rap1–MAPK and CaMKIV–CREB pathways, respectively. These findings may have implications in the general mechanisms of long-term synaptic modulation by neurotrophins.

Introduction

Remarkably, neurons have the ability to undergo long-lasting changes in the strength and pattern of their synaptic connectivity in response to environmental stimuli. Long-term modification of synapses is thought to mediate many aspects of brain functions, particularly learning and memory (Goelet et al., 1986). In general, long-term synaptic plasticity has two distinct features. First, long-lasting changes in the structure of synapses accompany alterations in synaptic efficacy that include both the modification of existing synapses and the formation of new synapses. Second, long-term synaptic plasticity requires changes in gene expression and new protein synthesis (Davis and Squire, 1984; Yin and Tully, 1996). The transcription factor cAMP response element (CRE)–binding protein (CREB) is thought to play a key role in the synthesis of new proteins required for long-term synaptic plasticity.

CREB, which is a member of basic leucine zipper (bZIP) family of dimeric transcription factors, controls the expression of many plasticity-related genes through binding to the CRE. Numerous studies have established that CREB plays a critical role in long-term synaptic plasticity and memory in a variety of model systems, such as flies, mollusks, and rodents (Silva et al., 1998; Kandel, 2001; Lonze and Ginty, 2002; West et al., 2002; Deisseroth et al., 2003). Is CREB involved in both structural and functional modifications of synapses? Inhibition of CREB activity in Aplysia blocks both long-term facilitation of synaptic efficacy and synaptic growth (Bartsch et al., 1995). In Drosophila melanogaster, however, CREB controls only functional, and not structural, synaptic plasticity (Davis et al., 1996). Gene knockout experiments in mice have yielded variable results regarding hippocampal synaptic plasticity (Murphy and Segal, 1997).

Correspondence to Bai Lu: bailu@mail.nih.gov

Abbreviations used in this paper: AChR, acetylcholine receptor; bZIP, basic leucine zipper; CAM, cell adhesion molecule; CaMK, calmodulin-dependent kinase; CRE, cAMP response element; CREB, CRE-binding protein; Dn, dominant-negative; ESC, evoked synaptic current; FRET, fluorescent resonance energy transfer; LTP, long-term potentiation; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate receptor; PKA, protein kinase A; ROI, region of interest; SYP, synaptophysin.

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Recently, neurotrophins have emerged as major factors that regulate synaptic structure and function (Poo, 2001; Lu, 2003; Lu and Je, 2003). These factors are widely expressed in the brain, and can elicit both acute and long-term modifications of synapses (Lu, 2004). In general, neurotrophins bind to their cognate Trk receptor tyrosine kinases and initiate their synaptic functions through the following three signaling pathways: MAPK, phosphatidylinositol 3-kinase, and PLC-γ (Kaplan and Miller, 2000; Huang and Reichardt, 2003). An important task is to determine the signaling mechanisms for specific neurotrophins in specific aspects of synaptic modulation. Several lines of evidence suggest that long-term synaptic modulation by neurotrophins also involves synaptic growth and requires new protein synthesis (Lu, 2004). Long-term treatment of hippocampal slices with brain-derived neurotrophic factor increases the number of synapse, spine density, and synaptic proteins in rodent slices with brain-derived neurotrophic factor increases the number of synapse, spine density, and synaptic proteins in rodent CA1 pyramidal neurons (Tartaglia et al., 2001; Tyler and Pozzo-Miller, 2001). In cultured Xenopus laevis neuromuscular synapses, long-term exposure to neurophin-3 (NT-3) induces a series of profound structural changes, specifically, an increase in the number and size of synaptic varicosities (Wang et al., 1995). In a recent study, we demonstrated that the long-term structural changes at the neuromuscular synapses induced by NT-3 require protein synthesis in the presynaptic neurons (Je et al., 2005).

Does long-term synaptic modulation by neurotrophins require the transcription factor CREB? What are the relationships between structural and functional changes at synapses induced by neurotrophins? In this study, we used the X. laevis neuromuscular synapses as a model system to study the signaling mechanisms underlying the structural and functional plasticity induced by NT-3. We demonstrated that although NT-3–induced enhancement in the efficacy at a single synapse requires CREB activation in presynaptic neurons, the long-term increase in the number of synaptic sites elicited by NT-3 is CREB-independent. Instead, the morphological changes in the presynaptic terminals require activation of the MAPK pathway. Thus, the structural and functional synaptic modifications by NT-3 are mediated by separate mechanisms. Using various imaging tools, we have also identified signaling events upstream of CREB and MAPK. Together, these data argue that a concomitant activation of CaMKIV–CREB–mediated transcription and sustained Rap1–MAPK signaling may be necessary for the long-term functional and structural changes necessary for neurotrophin-dependent synaptic modulation.

Results

Requirement of CREB activation in NT-3-induced long-term synaptic potentiation

CREB activation is critical for several forms of sustained synaptic plasticity (Kandel, 1997, 2001; Silva et al., 1998). It is unclear, however, whether CREB mediates the long-term synaptic modulation by neurotrophins. To measure CREB activation, we used an antibody that detects the phosphorylation at serine 133 of CREB. X. laevis spinal neurons exhibited rapid activation of CREB (<30 min) after NT-3 treatment (5 ng/ml), as indicated by dark nuclear staining (Fig. 1 A). To better quantify CREB activation, we used immunofluorescence, and measured the peak fluorescence intensity in the nuclear region (Fig. 1, B and C, and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200603061/DC1). Compared with untreated neurons, NT-3 treatment increased the level of fluorescence signals in the nucleus of spinal neurons by threefold (Fig. 1 C).

CREB can be phosphorylated on serine 133 by multiple kinases, including protein kinase A (PKA), calcium/calmodulin-dependent kinases (CaMKs), and MAPK. To identify the signaling pathways mediating the CREB activation, we examined phospho-CREB (pCREB) fluorescence in cultured spinal neurons in the presence of inhibitors specific for these pathways. Pretreatment of spinal neurons with the MAPK kinase (MEK) inhibitor PD098059 (10 μM; Alessi et al., 1995) or PKA inhibitor Rp-cAMP (5 μM; Matthies and Reymann, 1993) for 1 h could not attenuate the increase in pCREB fluorescence after NT-3 application (Fig. 1 C). In contrast, pretreatment with the CaM kinase inhibitor KN93 (1 μM; Corsi et al., 1998) completely prevented the increase in pCREB fluorescence induced by NT-3 (Fig. 1 C). These data suggest that CREB activation, at least within 1 h after NT-3 application, is mediated through the CaMKs.

It has been reported that in hippocampal neurons, CaMKIV is responsible for early activation of CREB, whereas the late, sustained phosphorylation of CREB depends on MAPK activity (Wu et al., 2001b). To examine whether CaMK or MAPK mediates the sustained CREB phosphorylation in developing spinal neurons, we monitored the time course of CREB activation in the presence or absence of specific inhibitors. NT-3 treatment significantly increased CREB phosphorylation as early as 30 min, and maximum activation of CREB was observed 1 h after NT-3 treatment (P < 0.05; Fig. 1 D). This increase was maintained at 93% level until 24 h after NT-3 treatment, indicating that NT-3 induced a sustained phosphorylation of CREB. The sustained activation of CREB was abolished by KN93, but not PD098059 (Fig. 1 D). Thus, in developing spinal neurons, NT-3–induced CREB phosphorylation is mediated by CaMKs, not MAPK.

NT-3 is known to induce both acute and long-term changes in synaptic efficacy at the neuromuscular synapses (Wang et al., 1995; Liou and Fu, 1997; Liou et al., 1997; Lu and Je, 2003). To determine whether CREB activation is necessary for either the acute or the long-term effects of NT-3, we introduced dominant-negative CREB (DnCREB) mutants into spinal neurons by embryonic injection. Two different DnCREB mutants were used; the K-CREB, a mutant that contains mutations in the DNA-binding domain of CREB; and the A-CREB, a mutant that contains basic-to-acidic residue mutations within the CREB bZIP domain (Walton et al., 1992; Ahn et al., 1998). These variants of CREB proteins act as dominant repressors for gene transcription by forming an inactive dimer with endogenous CREB and blocking its ability to bind cAMP response element (CRE). Therefore, expression of these DnCREB mutants cannot change the phosphorylation status of Ser133 in the endogenous CREB, but can still inhibit CREB-mediated gene transcription. The mRNAs of DnCREB mutants were co-injected with GFP mRNAs into
one blastomere of *X. laevis* embryos at the two-cell stage. Nerve-muscle cocultures were prepared from the injected embryos after 24 h. Expression of K-CREB in spinal neurons had no effect on the basal synaptic transmission. The frequency of spontaneous synaptic currents (SSCs) recorded from K-CREB–positive (N+) and K-CREB–negative (N–) neurons were 4.7 ± 0.5 events/min (n = 11) and 4.3 ± 0.6 events/min (n = 7), respectively. Moreover, identical to that observed in control synapses (N–), acute application of NT-3 induced an increase in frequency, but not amplitude, of SSCs (Fig. 2 A). Collectively, inhibition of CREB activation does not affect the acute modulation of transmission by NT-3.

In contrast, expression of K-CREB in presynaptic neurons completely blocked the enhancement of synaptic efficacy induced by long-term exposure to NT-3. In a culture treated with NT-3 for 48 h, the mean SSC frequency of the synapses made by K-CREB–expressing neurons was reduced to the level of NT-3–untreated neurons (Fig. 2 B). However, expression of K-CREB in the postsynaptic muscle cells had no effect on NT-3–induced long-term synaptic potentiation (Fig. 2 B), suggesting that CREB activation is only required in presynaptic neurons. Moreover, expression of K-CREB either pre- or postsynaptically had no effect on SSC amplitude, whether the synapses were treated with NT-3 or not (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200603061/DC1). Almost identical results were obtained from synapses expressing A-CREB presynaptically (Fig. 2 C). In addition to potentiating spontaneous synaptic activity, long-term treatment with NT-3 enhances functional synaptic transmission, as reflected by an increase in the amplitude of evoked synaptic currents (ESCs), which are elicited by stimulating presynaptic somata of spinal neurons (Je et al., 2005). Expression of K-CREB in presynaptic spinal neurons completely blocked the increase in ESC amplitude induced by NT-3 (Fig. 2 D). Collectively, these results suggest that presynaptic activation of CREB is required for NT-3–induced long-term, but not acute, synaptic transmission at the neuromuscular synapses.

**Long-term physiological effect of NT-3 mediated by the CaMKIV–CREB pathway**

Given that CaMK signaling was required for NT-3–induced CREB activation (Fig. 1, C and D), we next asked whether the CaMK pathway also mediates the long-term effects of NT-3. Treatment with NT-3 for 48 h increased the frequency of SSCs by ~2.5-fold over untreated control cells (Fig. 3 A). In contrast, in cultures pretreated with 1 μM KN93, NT-3 elicited no increase in SSC frequency, indicating that NT-3–induced synaptic potentiation requires activation of the CaMK signaling pathway. Inhibition of either MAPK (Je et al., 2005) or PKA (Fig. S2 D) did not exhibit statistically significant change in NT-3–induced long-term physiological effects. Therefore, these data suggest that the NT-3–induced long-term physiological effect is primarily mediated through a CaMK-dependent mechanism.

KN93 is a general inhibitor of CaMK family members, which include CaMKI, CaMKII, and CaMKIV, but CaMKI and CaMKII are enriched in neuronal processes and synapses,
whereas CaMKIV is relatively enriched in the nucleus (Deisseroth et al., 1998; Finkbeiner et al., 1997; Picciotto et al., 1995). The nuclear localization of CaMKIV suggests its ability to link extracellular signals to CREB activation in the nucleus. Therefore, we tested whether CaMKIV mediates both CREB activation and the long-term synaptic effects elicited by NT-3 by using Dn-CaMKIV. This mutant of CaMKIV bears a T196A mutation, which disrupts CaMKIV from binding to CaMKIV kinases (Tokumitsu et al., 1994). Expression of the Dn-CaMKIV mutant was reflected by GFP fluorescence in cultured spinal neurons (Fig. 3 B). NT-3 treatment for 30 min caused an approximately fourfold increase in pCREB immunoreactivity in control spinal neurons, but not in neurons expressing Dn-CaMKIV (Fig. 3 C). These findings are consistent with a recent study showing that acute application of NT-3 still elicits a rapid increase in the frequency of SSCs recorded from a synapse in which K-CREB is expressed presynaptically [N+]. [bottom] Summary of effect of acute application of NT-3 on control synapses (N–) without K-CREB, and synapses with presynaptic (N+) expression of K-CREB. Each data point represents normalized SSCs frequency (averaged from 10 min of recording) from a single synapse before and after NT-3 application. (B and D) Presynaptic, but not postsynaptic, expression of K-CREB prevents NT-3–induced long-term synaptic potentiation on SSC frequency (B) and ESC amplitude (D). M+, K-CREB expressed postsynaptically. (C) Presynaptic expression (N+) of A-CREB prevents NT-3–induced long-term synaptic potentiation. The number associated with each column represents the number of cells analyzed. Data are presented as the mean ± the SEM.

NT-3–induced translocation of GFP-CaM into nucleus
CaMKIV activation requires the translocation of Ca2+-calmodulin (CaM) into the nucleus, and such nuclear translocation of CaM appears to be important for the activation of CaMKIV–CREB pathway (Deisseroth et al., 1998). To test whether NT-3 is capable of inducing nuclear localization of CaM, we expressed GFP-CaM fusion protein in spinal neurons (Li et al., 1999). Expression of GFP-CaM did not affect the normal growth or morphology of
neurons (Fig. 4 A, left). Western blot showed the expression of GFP-CaM in *X. laevis* neural tubes 2 d after embryonic DNA injection. In embryos injected with GFP-CaM cDNA, a prominent band of 47 kD was detected using an anticalmodulin antibody. This band was also stained positively with antibody against GFP, confirming the expression of GFP-CaM fusion protein (Fig. 4 A, right).

We next studied the NT-3–induced movement of GFP-CaM in spinal neurons. GFP-CaM fluorescence was monitored in the nucleus and axonal periphery of neurons. Before application of NT-3, culture medium was replaced with Ringer’s solution for 2 h to reduce the background CaM movement caused by unspecified factors accumulated in the medium. Fluorescence intensity changes of selected areas in the nucleus and axons of a neuron (indicated by dotted lines in Fig. 4 B, top) upon NT-3 application were monitored by using time-lapse microscopy. Application of NT-3 evoked a rise in the fluorescence in the nucleus, paralleling a decrease in the fluorescence intensity in the axons (Fig. 4 B, top). The increase in nuclear CaM fluorescence was relatively fast; within 20 min, the nuclear fluorescence reached a plateau (Fig. 4 B, bottom left). In all experiments, the total averaged cellular fluorescence (corrected for photobleaching effects) remained unchanged, indicating that observed fluorescence intensity changes are caused by

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**Figure 3.** NT-3–induced CREB activation and long-term physiological change are mediated by CaMKIV. (A) Inhibition of CaMKs prevents NT-3–induced long-term synaptic potentiation. Cultures were treated with or without 1 μM KN93, which is a specific inhibitor for CaMKs. (B and C) Expression of Dn-CaMKIV abolishes CREB activation induced by NT-3. (B) Bright-field (top left), pCREB (top right, red), Dn-CaMKIV (bottom left, green), and merged fluorescence images (bottom right) of three spinal neurons, two of which express Dn-CaMKIV, as indicated by coexpression of GFP. After application of NT-3, the Dn-CaMKIV–negative neuron exhibit marked CREB phosphorylation (red). In contrast, the Dn-CaMKIV–expressing neuron (green) shows no CREB phosphorylation. Note that the neuron in the middle expresses a lower level of Dn-CaMKIV and shows a reduced CREB phosphorylation. Bar, 10 μm. White dashed lines indicate the outline of neuronal cell bodies. (C) Quantitative analysis of pCREB levels in Dn-CaMKIV (+) and (–) neurons before and after NT-3 application. (D and E) Inhibition of NT-3–induced long-term synaptic potentiation by presynaptic expression of Dn-CaMKIV. Dn-CaMKIV was expressed in spinal neurons. SSC frequency and ESC amplitude were measured in synapses treated with or without NT-3 for 2 d. (D) Effects on SSC frequency. (E) Effects on ESC amplitude. The number associated with each column represents the number of cells analyzed. Data are presented as the mean ± the SEM.
redistribution of GFP-CaM. Application of NT-3 for 30 min increased the intensity of nuclear fluorescence by 29%, but decreased that of the axonal fluorescence by 35%. These results indicate that application of NT-3 evoked active translocation of CaM from nonnuclear regions into nuclear region in neurons.

Long-term morphological effects of NT-3 mediated by MAPK pathway

In addition to physiological changes, long-term treatment with NT-3 induces the morphological development of presynaptic terminals (Liou and Fu, 1997; Wang et al., 1995). Using FM dye to label endocytosed synaptic vesicles, our previous study showed that chronic, but not acute, treatment with NT-3 promotes the formation of “synaptic varicosities,” which are enlargements along the axons that reflect clusters of synaptic vesicles and other presynaptic elements (Je et al., 2005). There are two potential problems associated with this approach. First, FM dye also labels postsynaptic membrane structures and organelles. To avoid this problem, we visualized the synaptic varicosity by expressing a fusion protein in which GFP is fused with the C terminus of synaptophysin (SYP-GFP), a major integral membrane protein of synaptic vesicles (Alsina et al., 2001; Ruthazer et al., 2006). SYP-GFP was coinjected with rhodamine-dextran in X. laevis embryos so that we could simultaneously label axonal morphology and synaptic varicosity (Fig. 5, A–C). Furthermore, to ensure that SYP-GFP labels functional synaptic vesicles, neurons transiently transfected with SYP-GFP were loaded with FM 4–64 dye. We found that that SYP-GFP fluorescence overlapped completely with FM dye–labeled structures at the presynaptic terminals (Fig. 6). The second problem is that labeling presynaptic vesicles alone may not faithfully reveal specific changes in the presynaptic terminals at the synapses. Therefore, we labeled postsynaptic acetylcholine receptors (AChRs) with a low concentration of Cy5-labeled α-bungarotoxin (Fig. 5 D, Cy5-α-BTX). The “synaptic site” was revealed by the juxtaposition of presynaptic SYP-GFP (green) and postsynaptic AChR (Fig. 5, E and F, red).

Using the double-labeling technique, we showed that chronic treatment (48 h) of NT-3 significantly increased the number of synaptic sites (Fig. 7 A). To test the role of CREB in NT-3–induced long-term morphological changes, we inhibited CREB activation by expressing K-CREB in spinal neurons and quantified the changes in synaptic sites. Treatment with NT-3 induced a twofold increase in the number of synaptic sites per myocyte. Surprisingly, although expression of K-CREB blocked the physiological changes of synapses triggered by NT-3, expression of K-CREB in spinal neurons could not inhibit a NT-3–induced increase in synaptic sites (Fig. 7 B). These results suggest that CREB activation is only required for functional, but not structural, changes of synapses induced by NT-3. Furthermore, these results raise the possibility of parallel signaling pathways mediating NT-3–induced functional and morphological changes.
What are the signaling pathways that mediate the increase in the number of synaptic sites induced by long-term exposure to NT-3? NT-3 is known to activate MAPK in X. laevis spinal neurons (Yang et al., 2001). Given that neurotrophins have been shown to promote neurite outgrowth via MAPK pathway in cortical neurons and PC12 cells (Markus et al., 2002), we sought to test whether MAPK activation is required for NT-3–induced increase in synaptic varicosity. Treatment with the MEK inhibitor U0126, which is known to block NT-3–induced MAPK activation (Yang et al., 2001), completely blocked changes in synaptic sites elicited by NT-3 (Fig. 7 C). However, neither the PKA inhibitor Rp-cAMP nor the CaMK inhibitor KN93 could block the NT-3 effect (Fig. 7 C). These results, together with the finding that MAPK is not involved in the long-term changes in synaptic efficacy induced by NT-3 (Je et al., 2005), reveal a complementary role of MAPK and CREB; MAPK mediates the morphological, whereas CREB mediates physiological, changes induced by NT-3.

Requirement of Rap1, but not Ras, for long-term morphological effects of NT-3
If CaMKIV is the upstream activator for CREB that mediates the functional changes of synapses upon NT-3 treatment, what is the upstream activator for MAPK that mediates the structural change of synapse induced by NT-3? In PC12 cells, Ras and Rap1, two related small GTP-binding proteins of the Ras subfamily, have been shown to mediate transient and sustained activation of MAPK, respectively (York et al., 1998). To monitor the kinetics of Ras and Rap1 activation by NT-3 in living spinal neuron, we used fluorescent resonance energy transfer (FRET) techniques using Raichu-Ras and -Rap1 constructs (Mochizuki et al., 2001). Raichu-Ras contains a pair of mutant fluorescence proteins (YFP and CFP) in its C terminus. Thus, activation of Ras caused by intramolecular binding of the Ras domain to the RafRBD domain brings CFP close to YFP, leading to FRET from CFP to YFP (Fig. 8 A). Raichu-Rap1 works very similarly, except Ras is replaced with Rap1. We found that
the activation of Ras differed from that of Rap1 in two ways. First, Ras activation, as reflected by the color change, was faster and more transient than Rap1 activation (Fig. 8, A [top] and B). Ras activation reached its maximum in $<10$ min and was diminished in $90$ min (Fig. 8 B). This may reflect the localization of Ras at the plasma membrane, where NT-3/TrkC signals were rapidly transduced (Fig. 8 B). In contrast, Rap1 activation was much slower and more sustained. Second, Ras activation was observed not only in the cell body, but also in the axonal processes. In contrast, Rap1 activation was concentrated primarily at the neuronal cell body and never extended to the axons (Fig. 8, A [bottom] and B). Thus, although Ras and Rap1 could both be activated by NT-3, they showed different spatial and temporal activation patterns.

Next, we asked whether the inhibition of Ras or Rap1 could block the long-term structural and functional change induced by NT-3. To test this, we expressed Dn-Ras or -Rap1 in X. laevis spinal neurons by embryo injection, and the nerve-muscle cocultures were treated with NT-3 for $2$ d. Expression of Dn-Ras and -Rap1 did not affect basal synaptic transmission (compare the white bars in Fig. 8 C). Moreover, long-term treatment with NT-3 induced a similar increase in SSC frequency in control neurons and neurons expressing either Dn-Ras or -Rap1 (Fig. 8 C and Fig. S2 C). However, expression of Dn-Rap1, but not Dn-Ras, completely blocked the long-term effect of NT-3 on synaptic sites (Fig. 8 D). These data, together with the spatiotemporal pattern of Rap1 activation induced by NT-3, suggested that the Rap1–MAPK pathway was responsible for NT-3–induced long-term structural, but not functional, changes at the neuromuscular synapses.

Discussion

It is well known that neuronal activity elicits long-term changes in the structure and function of synapses. Neurotrophins have recently emerged as a class of important regulators for synapse development and function. Neurotrophin-induced long-term synaptic changes resemble activity-dependent long-term synaptic plasticity in two fundamental ways: synaptic growth and dependence on protein synthesis (Lu, 2004). A question of general interest is whether neuronal activity and neurotrophins use the same or different molecular mechanisms to modulate synapses. In this study, we show that NT-3 induced two parallel molecular pathways. One involves the CaMKIV–CREB pathway, which is responsible for enhancement of synaptic efficacy, but not synaptic growth. The other involves Rap1–MAPK, which leads to increase in the number of synaptic sites, but not in synaptic transmission. To our knowledge, no study so far has demonstrated that neuronal activity triggers two parallel, but distinct, signaling pathways for structural and functional plasticity in vertebrate synapses. Thus, although similar molecules are used to mediate synaptic modulation induced by either neuronal activity or neurotrophins, the specific mechanisms underlying the two types of synaptic modulation are not the same.

The results obtained from this study, together with our previous findings and those of other laboratories, support a model in which neurotrophins, through activation of Trk receptor tyrosine kinases, induce long-term structural and functional changes at synapses through two parallel signaling pathways (Fig. 9). The Ca$^{2+}$–CaMKIV–CREB pathway ensures maturation of transmitter-release machinery for efficient functional transmission. The endocytosed Trk receptors and activation of Rap1–MAPK pathway promotes synaptic growth, leading
to the development of properly matched pre- and postsynaptic structures. For proper development of neuromuscular synapse in vivo, both pathways have to be activated in concert. Having one ligand to trigger both pathways synchronously is an ideal way to coordinate the structural and functional development of synapses. Similar mechanisms may also apply to synapse regeneration after injury. Local secretion of neurotrophins may ensure simultaneous morphological and physiological recovery of the injured synapses. Thus, concomitant activation of these two pathways via pharmacological means may help treatment of nerve injuries or other neurodegenerative diseases.

**CaMKIV-CREB pathway mediates NT-3-induced physiological changes at synapses**

The transcription factor CREB has been extensively studied for its role in long-term, activity-dependent modulation of synapses in both vertebrates and invertebrates. In invertebrates, CREB has been shown to play a critical role in the long-term facilitation (LTF) at the *Aplysia* sensory-motor synapses (Bailey et al., 1996). Moreover, Davis et al. (1996) have shown that CREB is involved in functional, but not structural, plasticity at the *D. melanogaster* neuromuscular synapses. At mammalian hippocampal synapses, early studies suggest that CREB is also essential for late-phase LTP (L-LTP; Bourchuladze et al., 1994).

In contrast, several recent studies indicated that CREB plays a more important role in cell survival than synaptic plasticity in the hippocampus (Pittenger et al., 2002; Balschun et al., 2003; Ramanan et al., 2005). However, expression of constitutively active CREB facilitates L-LTP, as well as long-term memory (Barco et al., 2002, 2005). Therefore, the specific role of CREB in activity-dependent synaptic modulation in vertebrate is an interesting subject that requires further investigation. We have now shown that CREB mediates the long-term changes in synaptic efficacy induced by NT-3. This is the first demonstration of the role of CREB in synaptic modulation by neurotrophins. Moreover, CREB is selectively involved in the physiological, but not morphological, changes of synapses induced by neurotrophins. These results suggest that activity-dependent and neurotrophin-induced synaptic modulation may use different molecular mechanisms, and provide new insights into a complex, but important, problem in the field.

In vitro studies have shown that CREB can be phosphorylated on Ser-133 by the following three signaling pathways: PKA, CaMKIV, and MAPK. However, specific pathways leading to CREB activation in neurons under physiologically relevant situations need to be worked out individually. Given that CREB is a key player in activity-dependent synaptic modulation, there has been considerable interest in determining the specific signaling events that activate CREB. Using cultured
hippocampal neurons, some studies showed that neuronal
activity rapidly induces a translocation of calmodulin from cy-
tosol to nucleus and activation of CaMKIV, leading to CREB
phosphorylation (Bito et al., 1996; Deisseroth et al., 1998;
Hardingham et al., 1999). In contrast, Impey et al. (1998) argued
that Ras-ERK-Rsk2 is more important than CaMKIV in activat-
ing CREB. Subsequent work by Wu et al. (2001b) suggested
that CaMKIV is responsible for the initial stage of fast CREB
activation (2–10 min after stimulation), whereas MAPK is
more effective in a later stage to promote a slow, but sustained,
CREB phosphorylation. However, an activity-dependent rise
in pCREB was greatly attenuated in both initial and late
stages in CaMKIV-deficient mice (Ho et al., 2000; Ribar et al.,
2000). In transgenic mice overexpressing Dn-CaMKIV, activity-
dependent activation of CREB was selectively blocked, whereas
that of MAPK was normal (Kang et al., 2001). These mice
exhibit selective impairments in L-LTP, but not early-stage
LTP, and in long-term memory, but not short-term memory.
These results suggest that CaMKIV is a predominant pathway
to CREB activation, and that the MAPK pathway can be sub-
sidary or dispensable.

What is the signaling event that activates CREB during
neurotrophin-induced synaptic potentiation? Our previous stud-
ies demonstrated that upon NT-3 application, there is a rise in
intracellular Ca²⁺, primarily through the release of Ca²⁺ from
IP3 receptor stores in X. laevis spinal neurons (He et al.,
2000). In transgenic mice overexpressing Dn-CaMKIV, activity-
dependent activation of CREB was selectively blocked, whereas
that of MAPK was normal (Kang et al., 2001). These mice
exhibit selective impairments in L-LTP, but not early-stage
LTP, and in long-term memory, but not short-term memory.
These results suggest that CaMKIV is a predominant pathway
to CREB activation, and that the MAPK pathway can be sub-
sidary or dispensable.

Another interesting finding is that inhibition of CREB
activity does not block the morphological changes in the nerve
terminals induced by NT-3. This segregates the structural
and functional alterations induced by the same neurotrophin.
In *Aplysia*, injection of oligonucleotides containing multiple
CRE-binding sites into the cell bodies of presynaptic sensory
neurons represses both structural and functional changes un-
derlying LTF (Bartsch et al., 1995). In contrast, CREB func-
tion is only required in change of synaptic strength, but not
synaptic growth, at the developing neuromuscular junction in
*D. melanogaster* (Davis et al., 1996). In the mammalian hippo-
campus, although the specific role of CREB in L-LTP remains
debatable, it is generally agreed that CREB is important for
long-term, activity-dependent modulation of synaptic efficacy
(Silva et al., 1998; Kandel, 2001; Lonze and Ginty, 2002; West
et al., 2002; Deisseroth et al., 2003). There is some evidence
that CREB is involved in long-term structural changes of hippo-
campus synapses, but in these cases CREB acts downstream of
hormones or trophic factors, rather than mediating the effects
of neuronal activity (Murphy et al., 1998; O’Connell et al.,
2000; Lee et al., 2004).

Why, then, is CREB involved in structural plasticity in
some systems, but not in others? One possibility is that the neu-
romuscular synapse is a unique system where CREB is not in-
volved in structural plasticity. Alternatively, studies on *Aplysia*
sensory motor synapses and hippocampal synapses deal with
synaptic plasticity in the adult, whereas those using *X. laevis*
nerve-muscle cocultures and *D. melanogaster* larvae NMJ re-
veal properties of developing synapses. A third possibility is
that at the neuromuscular junction, CREB is activated strictly
in presynaptic neurons, leading to transcription of genes impor-
tant for synaptic transmission and plasticity. In the hippocampal
neurons, CREB is activated largely in postsynaptic neurons.
It is conceivable that postsynaptic CREB activation may re-
sult in production of proteins involved in synaptic growth.
We favor a different interpretation; whether CREB is involved in morphological changes at synapses or not may represent a fundamental difference between activity-dependent and neurotrophin-induced synaptic modulation. In any case, separation of structural and functional plasticity offers a unique opportunity to study the molecular mechanisms underlying long-term synaptic modulation.

**Rap1–MAPK pathway mediates NT-3-induced morphological changes at synapses**

Numerous studies have revealed an undisputable role of MAPK in activity-dependent synaptic plasticity. In *Aplysia*, the activation of Aplysia MAPK, the homologue of Mawikish associated with and necessary for the establishment of LTP (Bailey et al., 1997). Substantial evidence indicates that MAPK is involved in both the induction and maintenance of L-LTP in the mammalian hippocampus (English and Sweat, 1996; Rosenblum et al., 2002; Kelleher et al., 2004). If activity-dependent and neurotrophin-induced synaptic modifications share similar molecular mechanisms, one would expect that MAPK be critically involved in synaptic potentiation induced by neurotrophins. Remarkably, MAPK is not required for the neurotrophin modulation of synaptic efficacy at the developing neuromuscular synapses. Instead, it plays a critical role in the formation of synaptic boutons induced by NT-3. Again, our data support two main points: (1) molecular mechanisms underlying activity-dependent and neurotrophin-induced synaptic modulation may be different; and (2) NT-3 triggers two parallel signaling pathways, which are separately responsible for structural and functional maturation of the synapses.

The separation of physiological and morphological effects of NT-3 on synapses is quite intriguing. There are several examples in which manipulations altered synaptic morphology, but not physiology, or vice versa. In one case, retina astrocytes secrete the extracellular matrix proteins thrombospondins, which increases the number of structural synapses that are functionally silent (Christopherson et al., 2005). Another example is that deletion of Munc18 in mice leads to a complete loss of functional synaptic transmission, but ultrastructurally these synapses are completely normal (Verhage et al., 2000). It is possible that morphological development of synapses is not necessary for simple spontaneous and evoked synaptic transmission, but may be needed for the full-bloom capacity, plasticity, or long-term stability of synapses. On the other hand, one may imagine that development of synaptic structures through Rap1–MAPK pathway may not be sufficient, and additional molecular and functional elements must be in place to achieve fully functional synapses.

An interesting finding was that Rap1, but not Ras, acts upstream of MAPK in mediating the increase in synaptic sites. How does Rap1 activation affect synaptic structure? One possibility is that only Rap1, but not Ras, mediates persistent MAPK activation, which may be required to trigger downstream signaling events necessary for structural plasticity. Parallel to this idea, previous studies indicate that NGF activates Ras through cell surface TrkA, leading to a transient activation of MAPK (York et al., 2000). In contrast, NGF-induced endocytosis of TrkA activates Rap1, resulting in sustained MAPK activity (Marshall, 1995; York et al., 2000; Wu et al., 2001a). Differences in the kinetics of MAPK activation may result in remarkably distinct cellular functions. In PC12 cells, transient activation of MAPK mediates proliferation, but sustained activation of MAPK induces differentiation (Marshall, 1995). We showed that application of NT-3 elicited a sustained activation of Rap1 localized largely in the cell body of spinal neurons, whereas Ras activation was transient and distributed throughout neuronal processes. Inhibition of Rap1, but not Ras, blocked the NT-3–induced MAPK activation and morphological changes of the nerve terminals. Rap1 may be involved in the signaling endosome to activate transcription. We have previously shown that endocytosis of NT-3 and its cognate receptor is required for NT-3–mediated long-term, but not short-term, synaptic modulation (Je et al., 2005). In PC12 cells, Rap1 and TrkA coprecipitate, and this signaling complex can affect MAPK activation (Wu et al., 2001a). Moreover, inhibition of receptor endocytosis attenuates activation of MAPK, but increases activation of other kinases (Wu et al., 2001a). Collectively, these results support the notion that the endocytosis of NT-3–TrkC complex results in a persistent activation of Rap1 and MAPK, which in turn trigger transcriptional activities that lead to the structural changes at the neuromuscular synapses.

An unsolved issue of Rap1–MAPK signaling in long-term synaptic modulation by neurotrophins is the identification of downstream targets of MAPK responsible for changing presynaptic morphology. One possibility is that the activation of MAPK affects cell adhesion molecules (CAMs), thereby modifying synaptic structures. The MAPK cascade has long been recognized as being required for growth factor–induced differentiation and outgrowth of nerve terminals (Pang et al., 1995; Perron and Bixby, 1999). In *Aplysia*, LTF elicits translocation of activated Aplysia MAPK into the neuronal nucleus and subsequent internalization of Aplysia CAM, a homologue of fasciclin II (FasII) in *D. melanogaster* and nerve CAMs in mammals (Mayford et al., 1992). Mutation in MAPK or inhibition of MAPK phosphorylation in Aplysia CAM blocks the internalization of Aplysia CAM, thereby preventing synaptic growth (Bailey et al., 1997; Martin et al., 1997). Similarly, at *D. melanogaster* NMJ, the modification of MAPK activity levels results in an altered number of synaptic boutons by affecting FasII localization at synaptic boutons (Schuster et al., 1996a,b). Therefore, neurotrophic activation of MAPK may affect cell adhesion molecules or other downstream proteins to remodel synaptic morphology. Another possibility is that neurotrophins may trigger MAPK to activate transcription factors other than CREB. Adf1, a member of the Myb-related family of transcription factors known to be activated by MAPK, has been shown to promote the development of synaptic morphology in *D. melanogaster* NMJ; genetic manipulation that decrease (or increase) the amount of Adf1 gives rise to a decrease (or increase) in synaptic boutons numbers in *D. melanogaster* NMJ, without affecting synaptic strength (DeZazzo et al., 2000). However, synaptic proteins under Adf1 regulation or upstream of Adf1 have not yet been identified. Therefore, it will be interesting to resolve downstream targets for neurotrophic activation of MAPK that mediate changes in synaptic morphology.
Materials and methods

DNA constructs, embryo injection, and X. laevis nerve-muscle coculture
dnCREB cDNA constructs (K- and A-CREB) were purchased from CLONTECH Laboratories, Inc. Dn-CaMKIV was a gift from M. Ehlers (Duke University Medical Center, Durham, NC). GFP-CAm expression vector was a gift from D. Chang (Hong Kong University of Science and Technology, Hong Kong, China). Dn-Ras and -Rap1a were gifts from A. Imanoto (University of Chicago, Chicago, IL). Raichu-Ras and Rap1a were obtained from M. Matsuda (Osaka University, Osaka, Japan). cDNAs were digested and subcloned into the expression vector pCDNA3.1 (+), which contains a T7 promoter for in vitro transcription of sense mRNAs. Capped mRNAs were generated by using the Message Machine kit (Ambion). X. laevis egg laying was induced by injecting female X. laevis with human chronic gonadotropin (Sigma-Aldrich). Resulting eggs were fertilized and cultured with sperm derived from male testes. mRNAs for NT-3, Dn forms of CREBs, Ras, and Rap1a, or CaMKIV were mixed with 1 μg/μl GFP mRNA at a 1:1 ratio. Approximately 6–12 nl of the solution was injected into one blastomere at the 2- or 4-cell embryonic stage using the Picopressure ejector (Parker Hannifin). 1 d after injection, the neural tube and associated myotomal tissues were dissected and used to prepare nerve-muscle cultures. Neural tube and associated myotomal tissue of X. laevis embryos at stage 20 were dissociated in Ca2+–Mg2+-free medium (58.2 mM NaCl, 0.7 mM KCl, and 0.3 mM EDTA, pH 7.4) for 15–20 min. Cells were plated on clean glass coverslips and grown in the presence or absence of NT-3 (2 nM; a gift from Regeneron Pharmaceuticals, Tarrytown, NY) for 2 d at room temperature, as previously described (Wang et al., 1995). The cultured cells were fixed with 4% paraformaldehyde (PFA) in 0.1% Triton X-100 in PBS for 5 min. Fixed cells were blocked with 5% nonfat milk and incubated with primary antibodies against phospho-CREB (Cell Signaling Technology) overnight at 4°C. Secondary antibody for immunofluorescence was Alexa Fluor 546 (Invitrogen). To visualize DAB staining, an ABC kit (Vector Laboratories) was used. The images were acquired using an Olympus IX70 microscope using a charge-coupled device camera, acquired with IPLab software and processed with Photoshop (Adobe).

FRET-based Raichu-Rap and -Ras imaging Confocal imaging was performed using an inverted LSM 510 laser scanning microscope and 40×, 1.3 NA, or 63×, 1.3 NA, oil-immersion objectives (all from Carl Zeiss Microimaging, Inc.). For imaging coexpression of YFP and GFP constructs, excitation lines of a blue diode laser of 405 nm for both CFP and YFP were used. Fluorescence was detected using a 458/ 514-nm dichroic beam splitter and a 470–525-nm bandpass filter for CFP and a 530–590-nm bandpass filter for YFP. In this way, any crosstalk and bleed-through of fluorescence were eliminated. Time-lapse scanning was performed with LSM 510 imaging system software. Post acquisition image processing was performed with the LSM 5 Image Browser (Carl Zeiss Microimaging, Inc.) and Photoshop 7.0 software (Adobe). X. laevis spinal neurons plated on a 35-mm diam coverslip were expressing pRaichu-derivised plasmids by embryonic injection. A phase-contrast image and fluorescent images for ECFP and EYFP were recorded every 20 s. Starting from 5 min, cells were stimulated with 10 ng/ml NT-3. We demonstrated cell images in intensity-modulated display (IMD) mode by MetaMorph software (Molecular Devices), according to the manufacturer’s instructions. In brief, background-subtracted images of EYFP and ECFP were first used for calculating the EYFP/ECFP ratio of each pixel. After determination of the upper and lower thresholds, the ratio value of each pixel was associated with one of six hues from black (low) to white (high). The intensity of each pixel was determined by the brightness of ECFP. Thus, in IMD mode, the hue and its intensity at each pixel represent the FRET efficiency and the probe concentration, respectively. The FRET images were collected every 20 s.

Electrophysiology

Cultured myotomal tissues were dissected and used to prepare nerve-muscle cultures. Neural tube and associated myotomal tissue of X. laevis embryos at stage 20 were dissociated in Ca2+–Mg2+-free medium (58.2 mM NaCl, 0.7 mM KCl, and 0.3 mM EDTA, pH 7.4). SSC frequency/amplitude was collected by a patch clamp amplifier (Axonpatch 200B; Axon Instruments), with a current signal filter set at 3 kHz. SSC frequency is defined as the number of SSC events per minute. The amplitudes of SSCs were analyzed using SCAN software (Dagan). To elicit ESSCs, square current pulses (0.5–1 ms; 0.5–5 V) were applied to the soma of spinal neurons with a patch electrode filled with Ringer’s solution at the neuronal soma under loose seal conditions (Yang et al., 2001). Pipette and membrane capacitance and seal resistance were compensated. For acute effect, the data from a 10-min recording before application of NT-3 was taken as “control,” and those from a 10-min recording after the increase has reached peak as “NT-3 treated.” For long-term effect, a 10-min mean volume of SSC frequency/amplitude taken from any parts of the recording from a synapse gave rise to very similar numbers. For the convenience of comparison, we averaged data from control synapses without NT-3 treatment (control), and normalized data from all other conditions to the control. The numbers in the graph bars represent the number of synapses examined (Figs. 2, 3, 5, 6, 8, S2, and S3).

Quantification of synaptic sites and FM dye imaging To visualize synaptic varicosity, we coinjected a small volume of a mixture, consisting of 2 μl of SYP-GFP plasmid DNA (1 μg/ml) and 2 μl of rhodamine-conjugated dextran dye (molecular weight, 10,000 Da), by embryo injection. SYP-GFP construct was a gift from J. Sullivan (University of Washington, Seattle, WA). To label postsynaptic AChRs, 100 nM Cy5-labeled rhodamine-conjugated dextran dye (molecular weight, 10,000 D), by em-
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