

# Intracellular $\text{Ca}^{2+}$ and $\text{Ca}^{2+}$ /Calmodulin-dependent Kinase II Mediate Acute Potentiation of Neurotransmitter Release by Neurotrophin-3

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**Abstract.** Neurotrophins have been shown to acutely modulate synaptic transmission in a variety of systems, but the underlying signaling mechanisms remain unclear. Here we provide evidence for an unusual mechanism that mediates synaptic potentiation at the neuromuscular junction (NMJ) induced by neurotrophin-3 (NT3), using *Xenopus* nerve-muscle co-culture. Unlike brain-derived neurotrophic factor (BDNF), which requires  $\text{Ca}^{2+}$  influx for its acute effect, NT3 rapidly enhances spontaneous transmitter release at the developing NMJ even when  $\text{Ca}^{2+}$  influx is completely blocked, suggesting that the NT3 effect is independent of extracellular  $\text{Ca}^{2+}$ . Depletion of intracellular  $\text{Ca}^{2+}$  stores, or blockade of inositol 1, 4, 5-trisphosphate (IP3) or ryanodine receptors, prevents the NT3-induced synaptic potentiation. Blockade of IP3 receptors can not prevent

BDNF-induced potentiation, suggesting that BDNF and NT3 use different mechanisms to potentiate transmitter release. Inhibition of  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII) completely blocks the acute effect of NT3. Furthermore, the NT3-induced potentiation requires a continuous activation of CaMKII, because application of the CaMKII inhibitor KN62 reverses the previously established NT3 effect. Thus, NT3 potentiates neurotransmitter secretion by stimulating  $\text{Ca}^{2+}$  release from intracellular stores through IP3 and/or ryanodine receptors, leading to an activation of CaMKII.

**Key words:** ryanodine receptors • inositol 1, 4, 5-trisphosphate receptors • acetylcholine • neuromuscular junction • synaptic transmission

## Introduction

Nerve growth factor, brain-derived neurotrophic factor (BDNF),<sup>1</sup> neurotrophin-3 (NT3), and NT4 belong to the neurotrophin family of signaling proteins essential for the survival and differentiation of different populations of neurons. Recent studies have revealed an important but previously unrecognized role of neurotrophins in synapse

transmission and plasticity (for reviews see Lu and Figurov, 1997; McAllister et al., 1999). In the central nervous system (CNS), the synaptic actions of neurotrophins have been studied mainly in the visual cortex and hippocampus. For example, BDNF and NT4, which activate TrkB receptor, but not NT3, which primarily activates TrkC receptor, have long-term modulatory effects on the formation of ocular dominance columns in the visual cortex (Cabelli et al., 1995, 1997; Riddle et al., 1995). In layer 4 and 6 of the visual cortex, BDNF and NT3 oppose each other in regulating the dendritic growth of pyramidal neurons (McAllister et al., 1995, 1997). In the hippocampus, BDNF has been shown to acutely regulate synaptic plasticity such as long-term potentiation (LTP) (Korte et al., 1995; Figurov et al., 1996; Patterson et al., 1996). In contrast, several recent reports have demonstrated that NT3 is not involved in the modulation of hippocampal LTP (Figurov et al., 1996; Kokaia et al., 1998; Ma et al., 1999). These studies suggest

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<sup>1</sup>*Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor;  $[\text{Ca}^{2+}]_i$ ,  $\text{Ca}^{2+}$  concentration(s); CaMKII,  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II; CNS, central nervous system; IP3, inositol 1, 4, 5-trisphosphate; NMJ, neuromuscular junction; NT, neurotrophin; SSC, spontaneous synaptic current; XeC, xestospongine C.

that the signaling mechanisms for BDNF and NT3 may be quite different.

Due to the complexity of CNS synapses, the mechanisms underlying the synaptic actions of neurotrophins are difficult to study. The neuromuscular junction (NMJ) offers a simple and easily accessible model to study the role and the mechanisms of neurotrophins in synaptic development and function in great detail. Two modes of neurotrophic regulation have been identified using the *Xenopus* nerve-muscle co-cultures: acute potentiation of neurotransmitter release and long-term regulation of synapse maturation. In the long-term mode, the spontaneous synaptic currents (SSCs) and impulse-evoked synaptic currents exhibit more mature properties after a prolonged treatment with NT3, and to a lesser extent, with BDNF (Wang et al., 1995; Liou and Fu, 1997; Liou et al., 1997). The neurotrophins induce an increase in the expression of synaptic vesicle proteins, and in the number of synaptic varicosities in the presynaptic site (Wang et al., 1995), as well as changes in the acetylcholine (ACh) receptors in the postsynaptic site (Wang and Poo, 1997; Gonzalez et al., 1999). In the acute mode, application of BDNF or NT3 rapidly enhances synaptic transmission at the NMJ (Lohof et al., 1993). The acute effect of neurotrophins is due strictly to an enhancement of transmitter release probability in the presynaptic site (Lohof et al., 1993; Stoop and Poo, 1995). The SSC frequency is markedly increased, whereas the quantal sizes are not affected. The expression of NT3, but not BDNF or NT4, in the postsynaptic muscle cells is activity-dependent (Xie et al., 1997). Further, the secretion of NT4 in muscle cells seems to be induced by repetitive stimulation of presynaptic neurons (Wang and Poo, 1997). These results suggest that neurotrophins may serve as target-derived, retrograde messengers that acutely modulate transmitter release at the developing neuromuscular synapses (Xie et al., 1997).

A critical and yet unresolved question is: what are the intracellular signaling mechanisms that mediate such rapid synaptic effects of neurotrophins? In the hippocampus, BDNF-induced enhancement of high frequency transmission at CA1 synapses appears to be mediated through the activation of mitogen-associated protein kinase and phosphatidylinositol-3 kinase pathways, but not phospholipase C- $\gamma$  pathway (Gottschalk et al., 1999). The acute modulation of synaptic transmission by BDNF at NMJ appears to require  $\text{Ca}^{2+}$  influx into the presynaptic terminals, but signaling events downstream of  $\text{Ca}^{2+}$  influx are not known (Stoop and Poo, 1996). Do neurotrophins share similar mechanisms in modulating synapses in the CNS and at the NMJ? Do BDNF and NT3 use the same signaling pathway to potentiate the neuromuscular synapses? In this report, we address the role of the nerve terminal  $\text{Ca}^{2+}$  in the acute regulation of neurotransmitter release at the NMJ by NT3. Specifically, we focus on the intracellular  $\text{Ca}^{2+}$  stores and the presynaptic  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII). A number of recent studies have suggested the involvement of intracellular  $\text{Ca}^{2+}$  stores in synaptic transmission (for review see Berridge, 1998). Although extensive studies have revealed diverse effects of CaMKII in postsynaptic functions (Chapman et al., 1995), the only clearly defined presynaptic effects of CaMKII is to regulate the availability of readily releasable synaptic vesicles

at the nerve terminals (Llinas et al., 1985; Greengard et al., 1993). We have now provided evidence that the acute potentiation of transmitter release by NT3 depends on a rise of  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) in the presynaptic terminals. Surprisingly, the increase in  $[\text{Ca}^{2+}]_i$  was due to  $\text{Ca}^{2+}$  released from intracellular stores, but not to  $\text{Ca}^{2+}$  influx from extracellular sources. Furthermore, the continuous activation of CaMKII, which is triggered by the increase in  $[\text{Ca}^{2+}]_i$ , appears to be required for the effect of NT3. These results may help understand how neurotrophins acutely modulate neurotransmitter release.

## Materials and Methods

### Embryo Injection

Specific peptide inhibitor for CaMKII (Ishida et al., 1995) (500  $\mu\text{M}$ ; Calbiochem) was mixed with rhodamine-dextran (10  $\mu\text{g}/\mu\text{l}$ , mol wt 10,000) at 1:1 ratio. Approximately 6–12 nl of the solution was injected into one blastomere of embryos at the 2- to 4-cell stage by a Picospritzer. The final concentration of the peptide within an injected blastomere was  $\sim 12.5 \mu\text{M}$ . 1 d after injection, the neural tube and the associated myotomal tissues were dissected and used to prepare nerve-muscle cultures. Cells containing CaMKII-pep were identified by rhodamine fluorescence.

### Culture Preparation

*Xenopus* nerve-muscle cultures were prepared according to the procedure described previously (Lu et al., 1992). In brief, the neural tube and the associated myotomal tissue of *Xenopus* embryos at stage 20 to 22 were dissociated in  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -free saline supplemented with EDTA (58.2 mM NaCl, 0.7 mM KCl, 0.3 mM EDTA, pH 7.4) for 15–20 min. The cells were grown on glass coverslips for 24 h at room temperature (20–22°C). The culture medium consisted (vol/vol) of 50% Leibovitz L-15 medium (Sigma), 1% FCS (Life Technologies), and 49% Ringer's solution (115 mM NaCl, 2 mM  $\text{CaCl}_2$ , 2.5 mM KCl, 10 mM Hepes, pH 7.6). NT3 ( $2\text{--}5 \times 10^{-9}$  M; kindly provided by Regeneron Pharmaceuticals, Inc.) and various inhibitors were applied directly to the culture media at the time of recording.

### Electrophysiology

Synaptic currents were recorded at room temperature in culture medium from myocytes innervated by spinal motoneurons using whole cell, voltage-clamp recording techniques (Lu et al., 1992). The solution inside the recording pipette contained: 150 mM KCl, 1 mM NaCl, 1 mM  $\text{MgCl}_2$ , and 10 mM Hepes buffer, pH 7.2. Membrane currents in all recordings were monitored by a patch clamp amplifier (EPC-7), with a current signal filter at 3 kHz. The membrane potentials of the muscle cells were generally in the range of  $-55$ – $-75$  mV and were voltage clamped at  $-70$  mV after measuring the membrane potentials. For experiments performed in the absence of external  $\text{Ca}^{2+}$ , the culture medium was replaced with a  $\text{Ca}^{2+}$ -free extracellular solution containing 115 mM NaCl, 2 mM  $\text{MgCl}_2$ , 10 mM Hepes, 3 mM EGTA, and 0.1% BSA. All data were stored on a videotape recorder for later playback on a storage oscilloscope (Tektronix TDS 420) and a chart recorder (Gould EasyGraf 240), or analysis using the SCAN program. To quantitatively measure the changes in neurotransmitter release, a time course of SSC frequency was first constructed on a minute-to-minute basis. The SSC frequencies in a 10-min period right before drug 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 drug application.

## Results

### $\text{Ca}^{2+}$ Influx Is Not Required for NT3-induced Synaptic Potentiation

We recorded synaptic activities at the neuromuscular synapses in 1-d-old *Xenopus* nerve-muscle cultures using whole

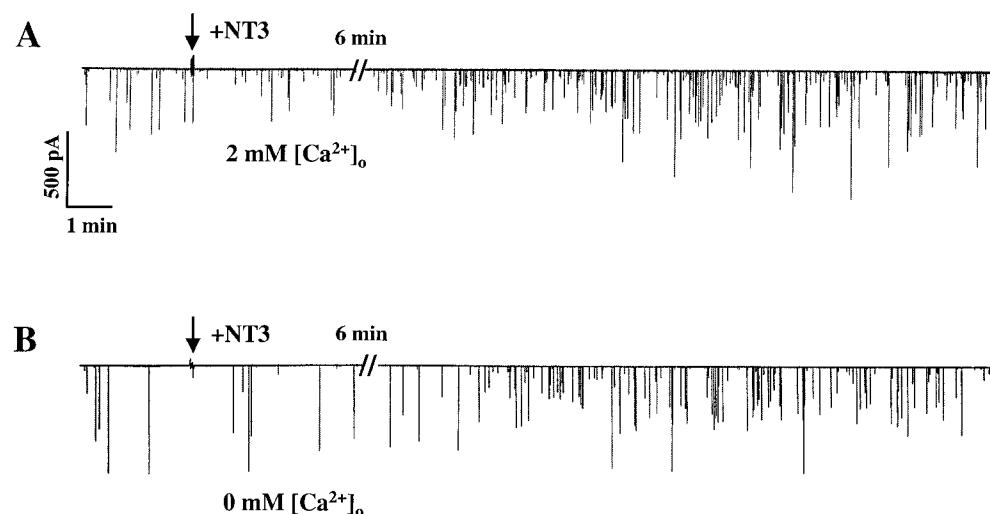
cell, voltage-clamp recording techniques. The SSCs are induced by spontaneous secretion of individual ACh-containing synaptic vesicles from motor nerve terminals independent of action potentials, since they are not affected in the presence of tetrodotoxin (Song et al., 1997). As shown before (Lohof et al., 1993; Stoop and Poo, 1995, 1996), acute application of NT3 (50 ng/ml) to the synapses in the presence of extracellular  $\text{Ca}^{2+}$  dramatically enhanced spontaneous transmitter release, as reflected by a rapid increase in the frequency of SSCs (Fig. 1 A). A previous report showed that in the same type of cultures, BDNF enhances synaptic transmission by facilitating  $\text{Ca}^{2+}$  influx into the presynaptic terminals (Stoop and Poo, 1996). We thus tested whether the acute NT3 effect uses a similar mechanism. The culture medium was substituted with  $\text{Ca}^{2+}$ -free extracellular solution after several washes with the same solution. Surprisingly, application of NT3 still elicited an increase in the frequency of SSCs under the zero external  $\text{Ca}^{2+}$  condition (Fig. 1 B and Fig. 2). The time courses of the NT3-induced increase in SSC frequency in both  $\text{Ca}^{2+}$ -free and  $\text{Ca}^{2+}$ -containing media were very similar, although the basal level of SSC frequency in  $\text{Ca}^{2+}$ -free media before NT3 application was slightly lower (Fig. 2, A and B). Quantitative analysis indicated that NT3 only increased the frequency, without affecting the amplitude or decay time of SSCs (data not shown), suggesting that NT3 facilitates presynaptic transmitter release in the absence of extracellular  $\text{Ca}^{2+}$ . To further examine the role of membrane  $\text{Ca}^{2+}$  channels, we blocked  $\text{Ca}^{2+}$  influx by the general  $\text{Ca}^{2+}$  channel blocker  $\text{Cd}^{2+}$  (0.4 mM). NT3 was still capable of elevating SSC frequency in the presence of  $\text{Cd}^{2+}$  (Fig. 2 B). Thus, NT3-induced potentiation of transmitter release does not depend on  $\text{Ca}^{2+}$  influx from extracellular sources. Our further analyses were thus performed mostly in  $\text{Ca}^{2+}$ -free medium.

### Role of Intracellular $\text{Ca}^{2+}$ Stores

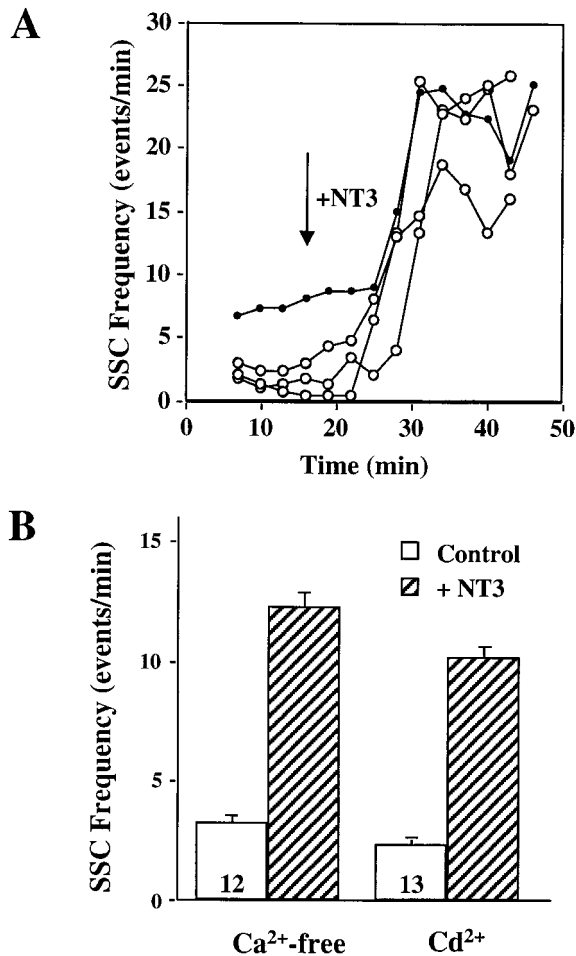
We next determined whether the NT3 effect is mediated by an increase in  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores. Thapsigargin inhibits  $\text{Ca}^{2+}$ -ATPase activity and

therefore has frequently been used to deplete all intracellular  $\text{Ca}^{2+}$  stores (Thastrup et al., 1990). Application of thapsigargin (2  $\mu\text{M}$ ) in both normal ( $n = 5$ , data not shown) and  $\text{Ca}^{2+}$ -free media (Fig. 3 A) elicited a transient increase in SSC frequency, which returned to control levels within 20–60 min. NT3 no longer elicited any changes in SSC frequency in thapsigargin-treated synapses (Fig. 3, A and B). When intracellular  $\text{Ca}^{2+}$  stores were depleted by thapsigargin, application of hypertonic solution (sucrose, 500 mM) to the synapses in  $\text{Ca}^{2+}$ -free medium still elicited a transient but marked increase in transmitter release, suggesting that there are still synaptic vesicles in the nerve terminals (data not shown). Thus, the lack of NT3 effect in thapsigargin-treated synapses was not due to vesicle depletion. These results suggest that an increase in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  release from intracellular stores may contribute to the facilitation of transmitter release by induced NT3.

There are two major pathways for the release of  $\text{Ca}^{2+}$  from intracellular stores: the inositol 1, 4, 5-trisphosphate (IP3) receptor and the ryanodine receptor (Berridge, 1998). Application of the IP3 receptor inhibitor xestospongin C (XeC; 1  $\mu\text{M}$ ) (Gafni et al., 1997) prevented the increase of SSC frequency elicited by NT3 (Fig. 3 B). The release of  $\text{Ca}^{2+}$  from IP3 receptors could further trigger  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from ryanodine receptors (Berridge, 1998). Low concentration of ryanodine (2.5–5  $\mu\text{M}$ ) may be used as a ryanodine receptor agonist, whereas high concentration (100  $\mu\text{M}$ ) may serve as an antagonist. In  $\text{Ca}^{2+}$ -free medium, application of ryanodine at high concentration (100  $\mu\text{M}$ ) had no effect on basal SSCs, but prevented the increase in SSC frequency induced by NT3 (Fig. 3 B) ( $P > 0.5$ , ANOVA). Pretreatment of the cultures with another ryanodine receptor antagonist 8-(dethylamino)octyl 3, 4, 5-trimethoxybenzoate (TMB-8) (30  $\mu\text{M}$ ) (Hunt et al., 1990) also blocked the NT3 effects (Fig. 3B). Unlike thapsigargin, however, ryanodine at lower concentration (2.5–5  $\mu\text{M}$ ) was not sufficient to elicit a consistent increase in SSC frequency (Fig. 3 C). Furthermore, application of NT3 in the presence of low concentration of ryanodine still elicited an increase in SSC frequency (Fig. 3



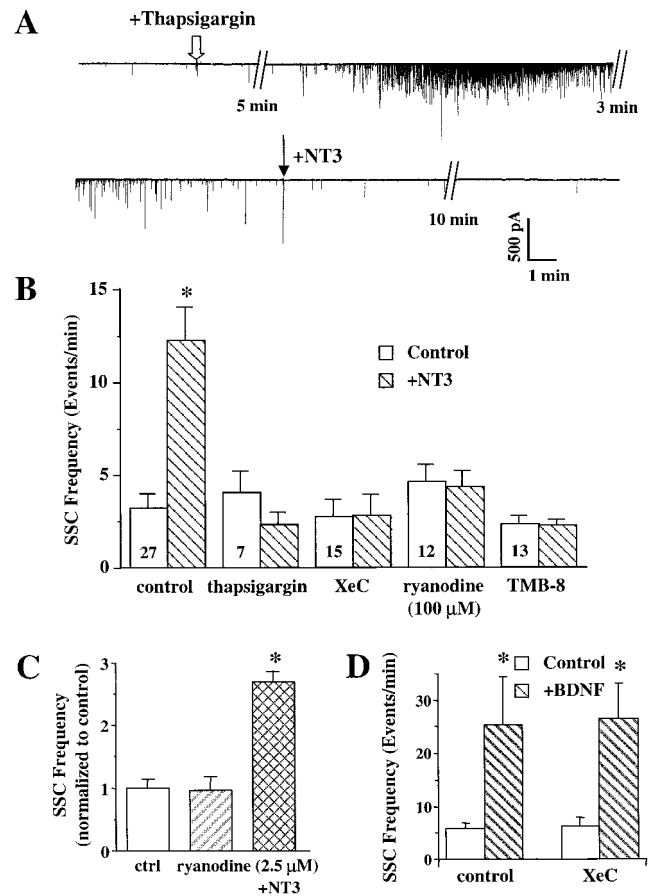
**Figure 1.** Acute potentiation of transmitter release at NMJ by NT3 is independent of extracellular  $\text{Ca}^{2+}$ . (A) A sample recording showing that application of NT3 (50 ng/ml) rapidly increases the frequency of SSCs in the normal culture medium. (B) Application of NT3 also rapidly increases the frequency of SSCs in the  $\text{Ca}^{2+}$ -free medium.



**Figure 2.** NT3-induced synaptic potentiation does not require Ca<sup>2+</sup> influx. (A) Time courses of NT3 effect on spontaneous synaptic activity in zero external Ca<sup>2+</sup> (open circles). The culture medium was replaced by Ca<sup>2+</sup>-free solution. The SSC frequency was monitored before and after NT3 application. Each point represents averaged SSC frequency in 3 min of recording. An example of recordings in normal medium (filled circles) is included for comparison. (B) The effect of NT3 on SSC frequency in Ca<sup>2+</sup>-free medium ( $n = 12$ ) or in medium containing the Ca<sup>2+</sup> channel blocker, Cd<sup>2+</sup> (CdCl<sub>2</sub>, 0.4 mM,  $n = 13$ ). For each synapse, a time course of SSC frequency was first constructed on a minute-to-minute basis. SSC frequencies are averaged from a 10-min recording right before NT3 application for controls, and a 10-min period starting from the highest number after NT3 application for NT3-treated groups. Error bars in this and all other figures are SEM. NT3 induced significant increases in SSC frequency in both conditions ( $P < 0.005$ ,  $t$  test). Similar methods were used to calculate averaged SSC frequencies in all other figures, unless indicated otherwise.

C). Thus, the synaptic action of NT3 is primarily mediated by the Ca<sup>2+</sup> release from IP<sub>3</sub> receptors, which further triggers Ca<sup>2+</sup> release from the ryanodine receptors.

We then tested whether BDNF, which requires Ca<sup>2+</sup> influx to enhance transmitter release (Stoop and Poo, 1996), also depends on Ca<sup>2+</sup> release from intracellular stores. Application of BDNF (50 ng/ml) elicited a fivefold increase in SSC frequency in normal Ca<sup>2+</sup>-containing medium (Fig. 3 D). In cultures pretreated with XeC (1 μM)



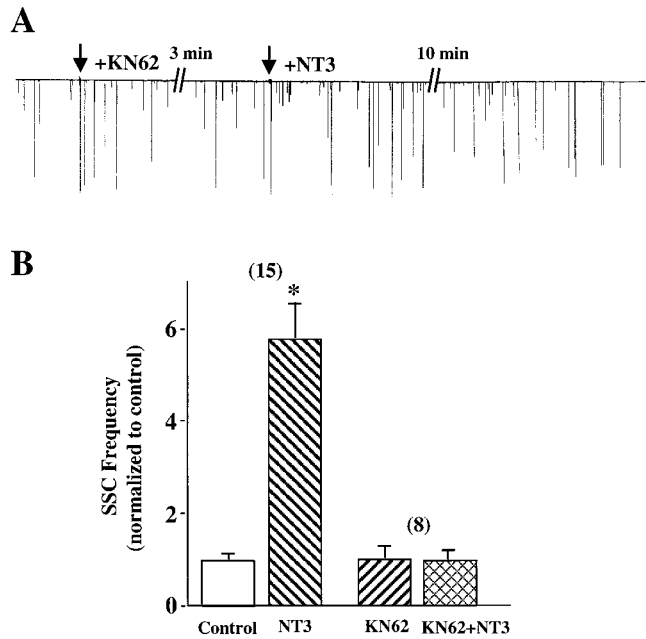
**Figure 3.** Enhancement of transmitter release by NT3, but not BDNF, requires Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores. All experiments were carried out in Ca<sup>2+</sup>-free conditions, except that shown in D. The cultures were incubated with various drugs for 15–60 min, and SSCs were recorded continuously to monitor the effects of the drug and NT3 application. Thapsigargin was used to deplete Ca<sup>2+</sup> from intracellular stores. Asterisk indicates data that are significantly different from the rest ( $P < 0.01$ , ANOVA test followed by post hoc comparison). (A) An example showing that thapsigargin (2 μM) prevents the NT3 effect. Application of thapsigargin (open arrow) elicited a transient but marked increase in SSC frequency due to the Ca<sup>2+</sup> release from intracellular stores. Application of NT3 after SSC frequency returned to normal could no longer increase the SSC frequency. (B) Summary of the drug effects. Final concentration of the drugs in the culture media: thapsigargin, 2 μM; XeC, 1 μM; TMB-8, 30 μM; and ryanodine as an antagonist, 100 μM. The number of synapses recorded are indicated in the control bar of each pair. Asterisk indicates  $P < 0.005$ ,  $t$  test. SSC frequencies are calculated in the same way as Fig. 2. (C) Summary of the effects of ryanodine as an agonist (2.5–5 μM). The SSC frequencies from a single synapse are counted for a 10-min period in control and a 10-min period after ryanodine application, and then a 10-min period after NT3 application. The data are then averaged and normalized to controls ( $n = 9$ ). Note that ryanodine at the low concentration could not block the NT3 effect. (D) BDNF-induced synaptic potentiation is independent of Ca<sup>2+</sup> release from intracellular stores through IP<sub>3</sub> receptors. BDNF was applied to culture dishes in the presence (right,  $n = 5$ ) or absence (left,  $n = 5$ ) of the IP<sub>3</sub> receptor inhibitor XeC (1 μM).

to block IP3 receptors, BDNF elicited the same magnitude of synaptic potentiation (Fig. 3 D), suggesting that the  $Ca^{2+}$  release from intracellular stores is not required for BDNF-induced synaptic potentiation. Thus, BDNF and NT3, two proteins from the same neurotrophin family, can both potentiate neurotransmitter release, but they use totally different intracellular mechanisms.

### The Effect of NT3 Requires Continuous Activation of CaMKII

The release of  $Ca^{2+}$  from intracellular  $Ca^{2+}$  stores induced by NT3 may trigger the activation of the presynaptic CaMKII. CaMKII has been shown to enhance transmitter release in adult squid giant synapses and mammalian brain synaptosomes, presumably due to an increase in the availability of readily releasable synaptic vesicles at the nerve terminals (Llinas et al., 1985; Nichols et al., 1990). We first tested whether CaMKII is involved in modulating transmitter release at the developing NMJ in the *Xenopus* culture system using KN62, a frequently used inhibitor for CaMKII (Tokumitsu et al., 1990). We found that bath application of KN62 (3  $\mu$ M) rapidly and reversibly reduced the amplitude of evoked synaptic currents. The average evoked synaptic current amplitudes before and 10 min after KN62 application were  $1.77 \pm 0.36$  nA and  $1.01 \pm 0.24$  nA, respectively ( $n = 8$ ,  $P < 0.01$ ,  $t$  test). In contrast, application of KN62 had little effect on the spontaneous release of neurotransmitters (Fig. 4, A and B). These results are consistent with the idea that CaMKII may regulate transmitter release when the terminal  $[Ca^{2+}]_i$  is elevated, but may not be very effective at the quiescent level of  $[Ca^{2+}]_i$ . Since NT3 acts presynaptically at the NMJ and CaMKII is capable of regulating transmitter release, we determined whether CaMKII is involved in the synaptic action of NT3 at the developing NMJ. Pretreatment of the nerve-muscle cultures with KN62 completely prevented the increase of SSC frequency elicited by NT3 (Fig. 4). The average SSC frequencies before and 10 min after KN62 remained unchanged (before KN62;  $5.9 \pm 0.6$  events/min; after KN62,  $5.6 \pm 0.6$  events/min;  $n = 8$ ,  $P > 0.1$ ). These results suggest that CaMKII is necessary for the NT3 regulation of neurotransmitter release.

KN62 may also inhibit other  $Ca^{2+}$ /calmodulin-dependent protein kinases (Enslin et al., 1994). To ensure that the NT3 effect is indeed mediated by CaMKII, we loaded a specific peptide inhibitor (CaMKII-pep) (Ishida et al., 1995) into the presynaptic neurons using embryo injection techniques (Alder et al., 1992; Lu et al., 1992). CaMKII-pep was injected together with rhodamine-dextran into one of the blastomeres of *Xenopus* embryos at the two-cell stage. The embryos were allowed to develop until stage 22 before being used to prepare the nerve-muscle co-cultures. Introduction of the CaMKII-pep did not appear to affect the development and morphology of the *Xenopus* embryos at the time of cell culture (not shown). The spinal neurons and the myocytes 1 d after cultures also exhibited normal morphology (Fig. 5 A). It has been shown that the rhodamine fluorescence faithfully reflects the cells containing the coinjected exogenous proteins (Alder et al., 1992; Lu et al., 1992). In cultures derived from injected embryos, substantial numbers of neurons and myocytes

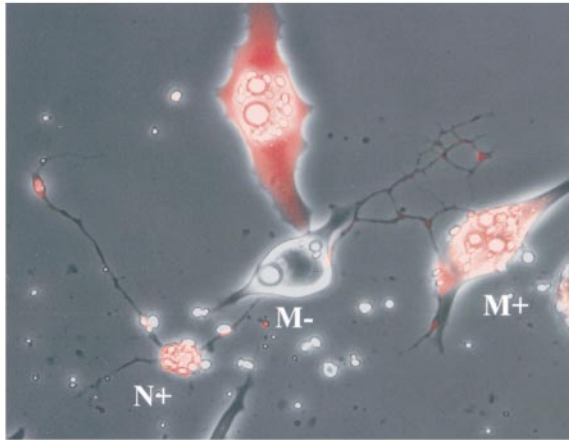
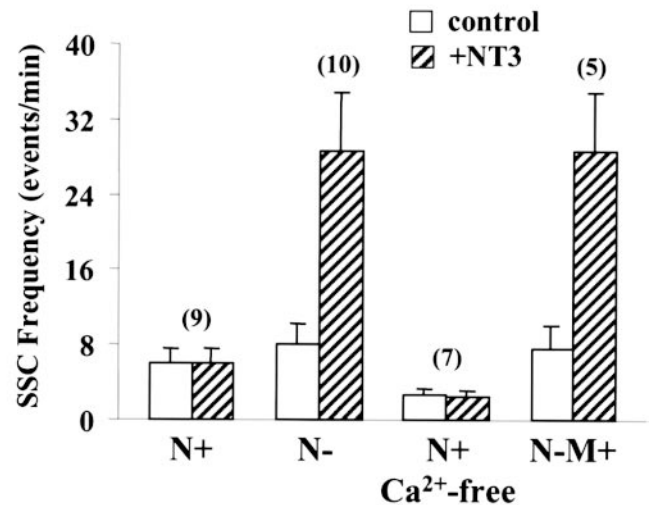
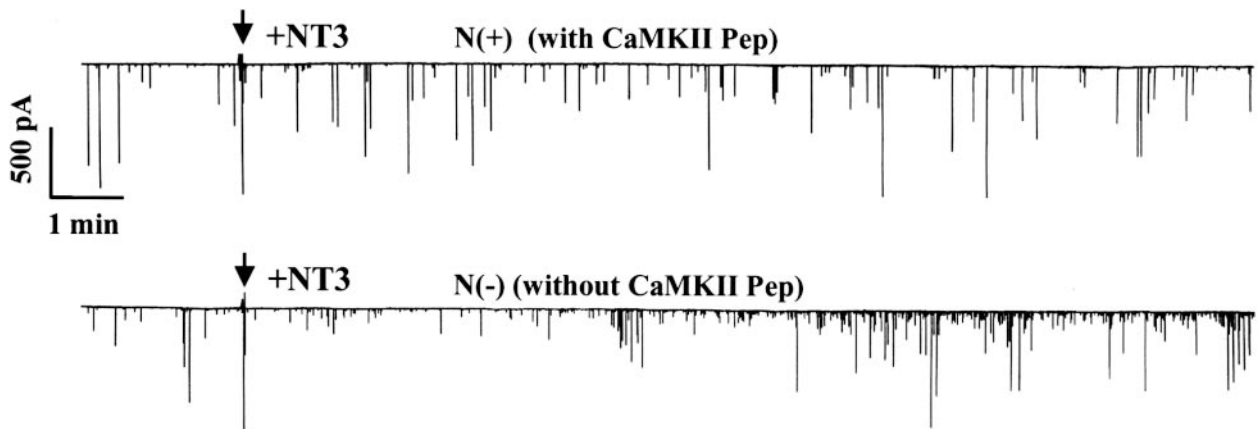


**Figure 4.** Effect of KN62 on NT3-induced synaptic potentiation. (A) A sample recording showing that pretreatment of the neuromuscular synapse with KN62 prevents the NT3-induced facilitation of transmitter release. (B) Blockade of NT3-induced synaptic potentiation of SSC frequency by KN62. In KN62 groups, the nerve-muscle cultures were pretreated with KN62 for 10–20 min before application of NT3. The number of synapses recorded are indicated above each pair of columns. In each experiment, SSC frequency was calculated from a 10-min recording period, and then the number after NT3 treatment was normalized to that before NT3 application. Asterisk indicates  $P < 0.01$ ,  $t$  test.

were CaMKII-pep-positive, as indicated by the rhodamine fluorescence (Fig. 5 A, N+ and M+).

Fig. 5 B shows SSCs recorded from a pair of synapses in which the presynaptic neurons were loaded with or without CaMKII-pep into normal  $Ca^{2+}$  medium, respectively. At the CaMKII-pep (–) synapse, application of NT3 still resulted in a marked increase in SSC frequency (Fig. 5, B and C). In contrast, loading of CaMKII-pep into the presynaptic neurons completely prevented the effects of NT3 (Fig. 5, B and C). Similar to KN62, loading of CaMKII-pep to the presynaptic neurons did not affect basal spontaneous transmitter release, but blocked the effect of NT3 (Fig. 5 C). Similar results were obtained in  $Ca^{2+}$ -free medium (Fig. 5 C). Furthermore, when CaMKII-pep was loaded into the postsynaptic myocytes (M+), NT3 was still capable of eliciting a significant increase in SSC frequency (Fig. 5 C). Taken together, these results strongly suggest that potentiation of transmitter release at the developing neuromuscular synapses by NT3 is achieved through the activation of CaMKII in the presynaptic neurons, but not the postsynaptic muscle cells.

Does the NT3-induced potentiation require a continuous activation of CaMKII? To address this question, we applied KN62 after synaptic transmission was potentiated by NT3. Fig. 6 A shows that within ~20–30 min after NT3 application, the increase in SSC frequency reached the peak. Application of KN62 at the peak gradually sup-

**A****C****B**

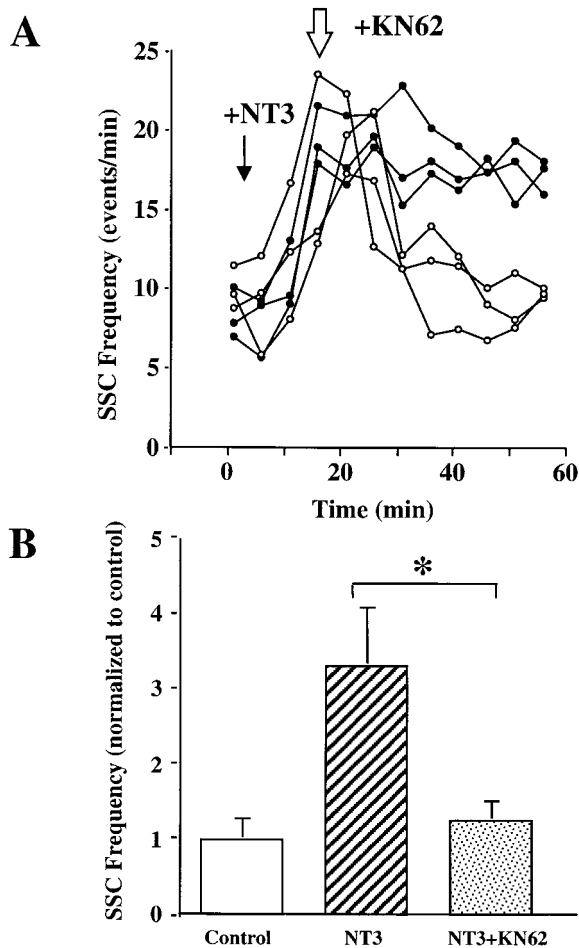
**Figure 5.** Inhibition of CaMKII prevents the effect of NT3. A peptide inhibitor for CaMKII (CaMKII-pep), together with rhodamine-dextran, was injected into *Xenopus* embryo at the two-cell stage, and the nerve-muscle co-cultures were prepared from the injected embryos. (A) Super-imposed phase and fluorescence micrographs of the 1-d-old nerve-muscle co-cultures showing cells loaded with (+) or without (-) CaMKII-pep. N, spinal neurons; M, myocytes. (B) A pair of recordings showing that loading of CaMKII peptide into presynaptic neurons N(+) prevented the NT3-induced increase in SSC frequency, whereas NT3 still enhanced transmitter release in a N(-) neuron in the same culture. (C) Summary of the effect of CaMKII-pep loaded into either presynaptic spinal neurons or postsynaptic myocytes. The number of synapses recorded are indicated above each pair of columns. In all N- conditions, application of NT3 elicited a significant increase in SSC frequency ( $P < 0.01$ ,  $t$  test).

pressed the SSC frequency. Quantitative analysis indicated that KN62 virtually reversed the NT3 effect (Fig. 6 B). Thus, continuous activity of CaMKII appears to be necessary for NT3 modulation of transmitter release at the developing neuromuscular synapses.

### Discussion

Previous work has shown that neurotrophins rapidly potentiate synaptic transmission through presynaptic mechanisms. The acute potentiation of transmitter release by BDNF is accompanied by a rise in  $[Ca^{2+}]_i$  in both the NMJ (Stoop and Poo, 1995) and at the CNS synapses (Berninger and Garcia, 1993; Marsh and Palfrey, 1996; Sakai et al., 1997; Li et al., 1998). However, it is unclear whether and how the increase in  $[Ca^{2+}]_i$  mediates the neurotro-

phin-induced synaptic potentiation and which downstream signaling events are involved. In this paper we report a surprising finding that the acute potentiation of transmitter release by NT3 at the neuromuscular synapses is independent of  $Ca^{2+}$  influx from extracellular sources. Instead, this potentiation is mediated by  $Ca^{2+}$  released from intracellular stores through IP<sub>3</sub> and ryanodine receptors. Thus, the mechanisms by which BDNF and NT3 modulate transmitter release could be quite different. Furthermore, we demonstrated that  $Ca^{2+}$  released from intracellular stores is capable of activating CaMKII, and the continuous activation of CaMKII is required for the effect of NT3. Taken together, this study provides, to our knowledge, the first evidence for a link between neurotrophins and CaMKII. These findings may provide new insights into the general mechanisms of neurotransmitter release and exocytosis,



**Figure 6.** Reversal of NT3 effects by addition of KN62 after NT3 application. (A) Examples showing the time courses that KN62 reverses the NT3 effect. NT3 was first applied (filled arrow) to the culture medium to elicit an increase in the frequency of SSCs. After the SSC frequency reached the peak, KN62 (open arrow) was either applied (open circles) or not applied (filled circles), and synaptic currents were continuously monitored. (B) Summary of the reversal effect of KN62. SSC frequencies are calculated in the same way as Fig. 3 C.  $n = 5$ . Asterisk indicates  $P < 0.01$ ,  $t$  test.

and how neurotrophic factors may regulate these processes.

In addition to extracellular  $Ca^{2+}$ , the release of  $Ca^{2+}$  from intracellular stores could either modulate or contribute directly to transmitter release (Berridge, 1998). Signals that result in the opening of either IP3 receptors or ryanodine receptors can generate local increases in  $[Ca^{2+}]_i$ , which in turn participates in the exocytotic process. Although still a fairly new concept, transmitter release triggered or modulated by the release of  $Ca^{2+}$  from intracellular stores has been shown in a number of systems such as the cholinergic synapse in *Aplysia*, reticulospinal synapse in lamprey, and sympathetic nerve terminals (Smith and Cunnane, 1996; Cochilla and Alford, 1998; Mothet et al., 1998). An important question then is whether neurotrophins, which are capable of eliciting an IP3 signal through the activation of phospholipase- $\gamma$  pathway (Segal and

Greenberg, 1996), can serve as endogenous neuromodulators to regulate synaptic transmission under physiological conditions. In this study, we have provided strong evidence that NT3 potentiates transmitter release by stimulating  $Ca^{2+}$  release from intracellular stores. We have shown that NT3 increased transmitter release in  $Ca^{2+}$ -free or  $Cd^{2+}$ -containing medium, and that pretreatment with thapsigargin prevented the NT3 effect. Moreover, inhibition of IP3 receptors blocked the NT3 effect. Thus, NT3 induces the release of  $Ca^{2+}$  through IP3 receptors at the terminals of developing spinal neurons, leading to an increase in spontaneous transmitter secretion. It is conceivable that similar mechanisms are used for NT3 to enhance evoked synaptic transmission, although we could not test this possibility because most of our experiments have to be done in  $Ca^{2+}$ -free medium. Consistent with our results, neurotrophins have been shown to induce an increase in  $[Ca^{2+}]_i$  in hippocampal neurons (Berninger and Garcia, 1993; Marsh and Palfrey, 1996), possibly by enhancing the release of  $Ca^{2+}$  from intracellular stores (Sakai et al., 1997; Li et al., 1998). The release of  $Ca^{2+}$  from IP3 receptors could further trigger  $Ca^{2+}$ -induced  $Ca^{2+}$  release from ryanodine receptors (Berridge, 1998). We found that the acute modulation of transmitter release by NT3 was blocked by the ryanodine receptor antagonist TMB-8 or a high concentration of ryanodine (100  $\mu$ M). However, activation of the ryanodine receptor alone by low concentrations of ryanodine (2.5–5  $\mu$ M) was not sufficient to enhance transmitter release, and application of NT3 on top of that still increased SSC frequency. Thus, both IP3 receptors and ryanodine receptors are involved in the acute effect of NT3, but the primary effect of NT3 is probably on the IP3 receptors. Although the electrophysiological analysis clearly indicates that NT3 potentiates neurotransmitter release through presynaptic mechanisms, our pharmacological experiments can not formally establish that NT3 acts on presynaptic terminals directly. We can not rule out the possibility that NT3 initially acts on postsynaptic muscle cells to trigger the release of  $Ca^{2+}$  from intracellular stores, leading to the secretion of some retrograde signal to activate presynaptic CaMKII.

Although our results suggest a role of  $Ca^{2+}$  release from internal stores in NT3-induced synaptic potentiation, a previous study has shown that the acute potentiation by BDNF in the same preparation requires external  $Ca^{2+}$  (Stoop and Poo, 1996). BDNF binds and interacts almost exclusively with the TrkB receptor, whereas NT3 binds primarily to the TrkC receptor (Kaplan and Stephens, 1994). It is possible that the activation of TrkB triggers  $Ca^{2+}$  influx, whereas that of TrkC is coupled to internal  $Ca^{2+}$  stores in the developing spinal neurons. Indeed, we found that inhibition of  $Ca^{2+}$  release from internal stores can not block the BDNF-induced synaptic potentiation. Similarly, both BDNF and NT3 attract growth cone turning in these developing spinal neurons, but the intracellular mechanisms that mediate the turning responses to the two factors are completely different (Song et al., 1998). The BDNF effect requires  $Ca^{2+}$  influx into the terminals and elevation of  $[cAMP]_i$ , whereas the NT3 effect is independent of extracellular  $Ca^{2+}$ . In this study, we show that the activation of IP3 receptors is required for the synaptic effect of NT3, but not for that of BDNF. Thus, although

both enhance synaptic transmission at developing neuromuscular synapses, the two factors may require  $\text{Ca}^{2+}$  from difference sources, one extracellular and one intracellular.

The potentiation of transmitter release usually occurs at least 5–10 min after NT3 application (Figs. 1, 2, and 6; see also Lohof et al., 1993; Xie et al., 1997). This time course implies that NT3-induced  $\text{Ca}^{2+}$  release modulates the transmitter release mechanisms, rather than contributing directly to the triggering of the exocytosis process. The NT3 modulation is known to be presynaptic in nature (Lohof et al., 1993). What are the presynaptic targets downstream of  $\text{Ca}^{2+}$  release induced by NT3? CaMKII may serve as an excellent candidate, because its role in transmitter release is relatively well-defined (Llinas et al., 1985; Lin et al., 1990; Nichols et al., 1990; Stanton and Gage, 1996; Jin et al., 1998; for review see Greengard et al., 1993). Extensive studies indicate that the activation of CaMKII is triggered by  $\text{Ca}^{2+}$  influx through extracellular sources. An important finding in this study is that CaMKII can also be activated by  $\text{Ca}^{2+}$  released from internal stores through IP<sub>3</sub> and ryanodine receptors. We showed that even in the complete absence of  $\text{Ca}^{2+}$  influx, the NT3-induced potentiation of transmitter release can be blocked by the CaMKII inhibitors CaMKII-pep or KN62. Furthermore, we found that KN62 can reverse established synaptic potentiation after NT3 application in  $\text{Ca}^{2+}$ -free medium. These results not only provide a link between internal  $\text{Ca}^{2+}$  stores and CaMKII activation, but also point to CaMKII as a downstream signaling mediator for NT3-induced synaptic potentiation. Since it is difficult to test whether the activation of CaMKII alone is sufficient to mimic the NT3 effect, we can not rule out the possible involvement of other processes that may also contribute to the NT3-induced synaptic potentiation.

The authors wish to express their gratitude to Drs. Doug Fields, Lucas Pozzo-Miller, Stanko Stojkovic, and members of the Lu laboratory for helpful discussions and critical comments on the manuscript. We would also like to thank Regeneron Pharmaceuticals for providing recombinant NT3.

Submitted: 16 February 2000

Revised: 11 April 2000

Accepted: 11 April 2000

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