Neurotrophin 3 potentiates neuronal activity and inhibits γ-aminobutyratergic synaptic transmission in cortical neurons

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ABSTRACT   Neurotrophins have traditionally been regarded as slowly acting signals essential for neuronal survival and differentiation. However, brain-derived neurotrophic factor and neurotrophin 3 (NT-3) have recently been reported to exert an acute potentiation of synaptic activity at the amphibian neuromuscular junction. Little is known about the role of neurotrophins on functional synapses in the central nervous system. Here we show that NT-3 rapidly increased the frequency of spontaneous action potentials, and it synchronized excitatory synaptic activities in developing cortical neurons. Moreover, the inhibitory synaptic transmission mediated by γ-aminobutyric acid (GABA) subtype A receptors was found to be reduced by NT-3. Thus, the excitatory effects of NT-3 on spontaneous action potentials were attributable to a reduction of GABAergic transmission. Our findings, together with previous reports of rapid regulation of central nervous system neurotrophin expression by neuronal activity and of the role of GABAergic transmission in cortical plasticity, suggest a mechanism for modulation of synaptic transmission and activity-dependent synaptic modulation in cortical neurons.

Extensive studies in the past few years have elucidated the important roles that the neurotrophins [including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5)] play in the development and maintenance of both peripheral and central nervous systems (for recent reviews see refs. 1 and 2). The functions of neurotrophins on distinct neuronal populations are mediated by the cellular expression of the trk family of tyrosine kinase receptors (3, 4). While the effects of neurotrophins on neuronal survival and differentiation are well established, little is known about their role in synaptic development and function. Moreover, the classic view of neurotrophins stresses their long-lasting, chronic actions. However, a recent study showed that BDNF and NT-3 rapidly enhance synaptic activity at developing neuromuscular junctions in *Xenopus* cell culture (5). Will neurotrophins also serve as neuromodulators exerting fast regulation on synaptic activity in central nervous system (CNS) neurons?

Neurotrophins and their receptors, the trk tyrosine kinases, are widely expressed in the brain (6–12). The fact that the expression of neurotrophins is rapidly enhanced by neuronal activity in CNS (13–22) suggests their role in activity-dependent processes, such as synaptic development and plasticity. In the present study, we sought to examine the acute effects of NT-3 on developing cortical synapses in culture, using electrophysiological approaches. We now provide direct evidence that NT-3 specifically enhances impulse activity in neurons derived from somoatosensory cortex and induces synchronization of excitatory synaptic currents. Moreover, the potentiation of neuronal activity appears to be attributable to a reduction of γ-aminobutyric acid (GABA)er-

gic synaptic transmission. Our results support the hypothesis that a reciprocal regulation between neurotrophin expression and neuronal activity may operate to modulate synaptic efficacy.

MATERIALS AND METHODS

Tissue Cultures. Dissociated cultures of cortical neurons were prepared as previously described (23). Briefly, cells from embryonic day 19 (E19) rat somatosensory cortex were dissociated and plated onto polylysine-coated dishes at a density of 0.5 million cells per 35-mm dish. They were grown in serum-free medium until use. In general, 2- to 3-week-old cultures were used for electrophysiological recordings.

Electrophysiological Recordings. Electrophysiological recordings were performed by using standard whole-cell patch clamp techniques (24, 25). During the experiment, the culture dish was continuously perfused with oxygenated extracellular (Ringer) solution at room temperature. The Ringer solution contained 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2.6 mM $NaHCO_3$, 2 mM $CaCl_2$, and 20 mM dextrose. It was bubbled with 95% O_2/5% CO_2 (pH 7.4). The continuous perfusion of the vehicle solution alone did not have any effects on the recording. In addition, we have routinely used bovine serum albumin (2–5 mg/ml) in the perfusate, and it never elicited any change in neuronal activity. NT-3 was applied through perfusion Ringer at a concentration of 5×10^{-10} M in this and all other experiments. Whole-cell electrodes were 5–10 mΩ in resistance and contained 120 mM potassium gluconate, 10 mM Hepes, 5 mM EGTA, 2 mM $MgCl_2$, 4 mM MgATP, 0.5 mM $CaCl_2$, and 0.3 mM GTP, pH adjusted to 7.2 with KOH. Dual cell recordings were performed to study evoked synaptic currents. Synaptically connected pairs of neurons were found by visual inspection and then tested by stimulating one neuron and recording from the other. The presynaptic neuron was current-clamped and depolarized to produce a single action potential in every 10 sec. The postsynaptic cell was voltage-clamped at different holding potentials to measure the reversal potential (V_{rev}). We routinely monitored the cell recorded for a long time before application of drugs. Cells with a resting membrane potential less than −45 mV or those that showed a change in membrane potential, input resistance, or a gradual rundown of postsynaptic current amplitudes were considered as unhealthy and discarded without further experimentation. For GABAergic synapses ($V_{rev} = −45$ mV), a cocktail of glutamate antagonists [50 μM 2-amino-5-phosphonovaleric acid (APV) for *N*-methyl-D-aspartate (NMDA) receptors and 5 μM 6,7-dinitroquinoxaline-2,3-

dione (DNQX) for non-NMDA receptors] was added to the perfusate to block the excitatory synaptic transmission, and inhibitory postsynaptic currents (IPSCs) were recorded from the postsynaptic neurons under voltage-clamped mode. The postsynaptic currents were recorded under a voltage-clamped mode. Data were collected by a patch clamp amplifier (Axoclamp-2B), stored in video tapes, and analyzed by P-Clamp software (Axon Instruments, Foster City, CA).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR). The RNAzol method (Biotecx Laboratories, Houston) was used to isolate total RNA from cultured cortical neurons (10^7 cells) and from whole adult rat brain (positive control). RT-PCR was performed using a standard protocol (26). Approximately 10 μg of total RNA from the above sources was reverse transcribed, using specific reverse primers for the messages of rat glyceraldehyde-phosphate dehydrogenase (GAPDH, control for PCR, low-affinity NGF receptor (p75), trkA, trkB, and trkC. After cDNA synthesis, the reaction mixtures were depleted of the oligonucleotides by using Centricon-100 devices (Amicon) and the volumes were restored to 500 μl with 1 mM Tris·HCl/0.1 mM EDTA, pH 8.0. An aliquot (62 μl) was used for each PCR (5 min at 96°C, 5 min at 55°C, and 40 min at 72°C for 1 cycle and 40 sec at 96°C, 2 min at 55°C, and 2 min at 72°C for 40 cycles). The specific primers used in this experiment are as follows: GAPDH-1, 5'-CTGGAGAAACCTGCCAAGTATG-3'; GAPDH-2, 5'-CACCCTGTTGCTGTAGCCATAT-3'; p75-1, 5'-GATATGGTGACCACTGTGATG-3'; p75-2, 5'-ATCAAGCGTTGCACTGTCCTG-3'; trkA-1, 5'-CCTGAG-GACCCCATCCCTG-3'; trkA-2, 5'-AGAACTGCCAC-CCAGTGTCATG-3'; trkB-1, 5'-TCCAAACACATGAAT-GAAACA-3'; trkB-2, 5'-GTGATTGGTGACGTGTAT-TTTG-3'; trkC-1, 5'-AACAGCAATGGGAACGCCAGC-3'; and trkC-2, 5'-TTGGGAGCCATCAGCACTGATG-3'.

Materials. Purified human recombinant NT-3 was provided by Genentech. k252a was from Biomol (Plymouth Meeting, PA). DNQX was from Research Biochemicals (Natick, MA). All other reagents and chemicals were from Sigma.

RESULTS

The acute effect of neurotrophins on neuronal activities was examined by using whole-cell, voltage- or current-clamped recordings in cultured neurons from the rat somatosensory cortex (24, 25, 27). Application of NT-3 (5 × 10^{-10} M in all experiments) significantly elevated the frequency of spontaneous action potentials (spike frequency) and synchronized excitatory synaptic activities. In a current-clamped neuron, NT-3 not only increased the number of action potentials but also induced bursts of spikes reminiscent of those caused by synchronized excitatory synaptic activity (Fig. 1A, upper trace). We compared the spike frequency before and after NT-3 treatment for each of the 24 cells recorded, and we found that NT-3 induced a statistically significant increase in 17 cells. Virtually all cells (15/17) that responded to NT-3 showed clusters of excitatory synaptic events underlying the bursts of action potentials, while the control recording showed no clusters. The NT-3-induced synchronization of synaptic activities can be seen more clearly in Fig. 1B, which shows both spontaneous and evoked excitatory postsynaptic currents (EPSCs) recorded from a voltage-clamped postsynaptic neuron. The evoked EPSCs were elicited by stimulating one of the presynaptic neurons to fire a single action potential (marked by an asterisk). The clusters of both spontaneous and evoked EPSCs were much longer in duration and consisted of many synaptic events in the presence of NT-3, suggesting that synaptic activities were synchronized (Fig. 1B).

The spike frequency consistently increased after NT-3 treatment. In contrast to the action of neurotrophins on
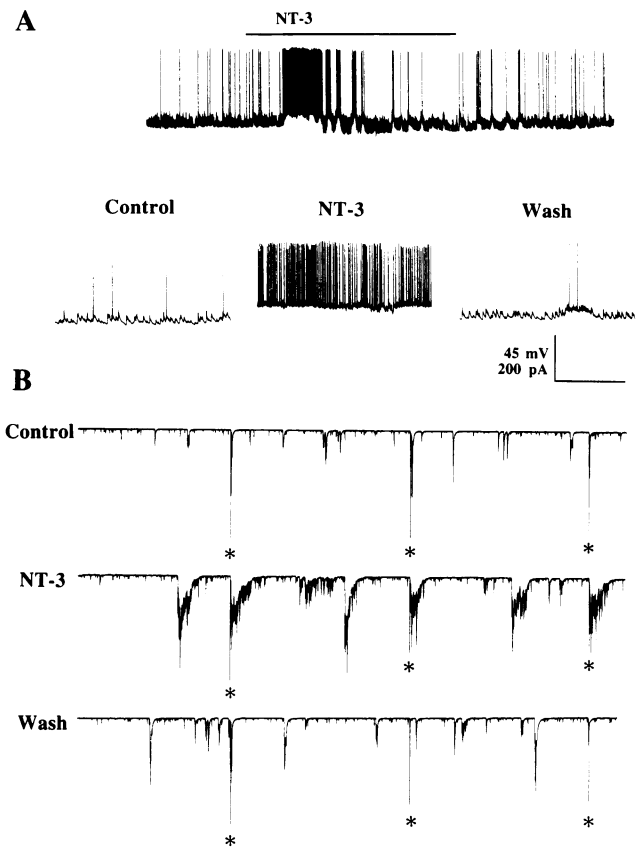

FIG. 1. Potentiation of neuronal activity by NT-3. (A) Voltage traces recorded from a cortical neuron under a current-clamped mode. Treatment of NT-3 markedly increased the spike frequency. Lower traces show the responses at a faster time scale to accentuate individual action potentials. Notice the clusters of synaptic events underlying the bursts of action potentials after the application of NT-3. Resting membrane potential = −72 mV. Fourteen-day culture. Time bar = 25 sec for the upper trace and 3 sec for lower traces. (B) Current traces of a voltage-clamped neuron from a paired recording. In the presence of NT-3, synaptic currents were synchronized, producing a bursting synaptic pattern in both evoked (indicated by asterisks) and spontaneous events. The NT-3 trace was taken 3 min after NT-3 application, and the wash, 10 min after washout of NT-3. V_{hold} = −80 mV. Twenty-day culture. Time bar = 4 sec.

neuronal survival and differentiation, the potentiation of neuronal activity by NT-3 was very rapid; the frequency peaked in a minute or two after NT-3 application (Fig. 2A). The effects were often transient, and the frequency of action potentials returned to control levels even in the presence of the trophic factor (Fig. 1A; Fig. 2A, filled symbol curves). Unlike the spike frequency, neurotrophin modulation of synchronized synaptic events persisted as long as NT-3 was present. The rapid onset of NT-3 actions represents a mode of the neurotrophin function that differs from the traditional differentiation effects.

The changes of spike frequency under various treatment conditions are summarized in Fig. 2B. The cortical neurons recorded showed a substantial increase in spike frequency after NT-3 treatment (5.01 ± 0.76 times control, mean ± SEM, $n = 10$, $P < 0.001$). In contrast, the treatment with protamine, a protein that has physicochemical properties similar to those of neurotrophins, did not elicit any significant change in spike frequency (0.90 ± 0.12, $n = 6$). Pretreatment for 1 hr with k252a, a specific inhibitor for the trk receptor tyrosine kinases (28–30), blocked the effects of NT-3 (0.88 ± 0.08, $n = 8$) (Fig. 2B). Therefore, the neurotrophin effects
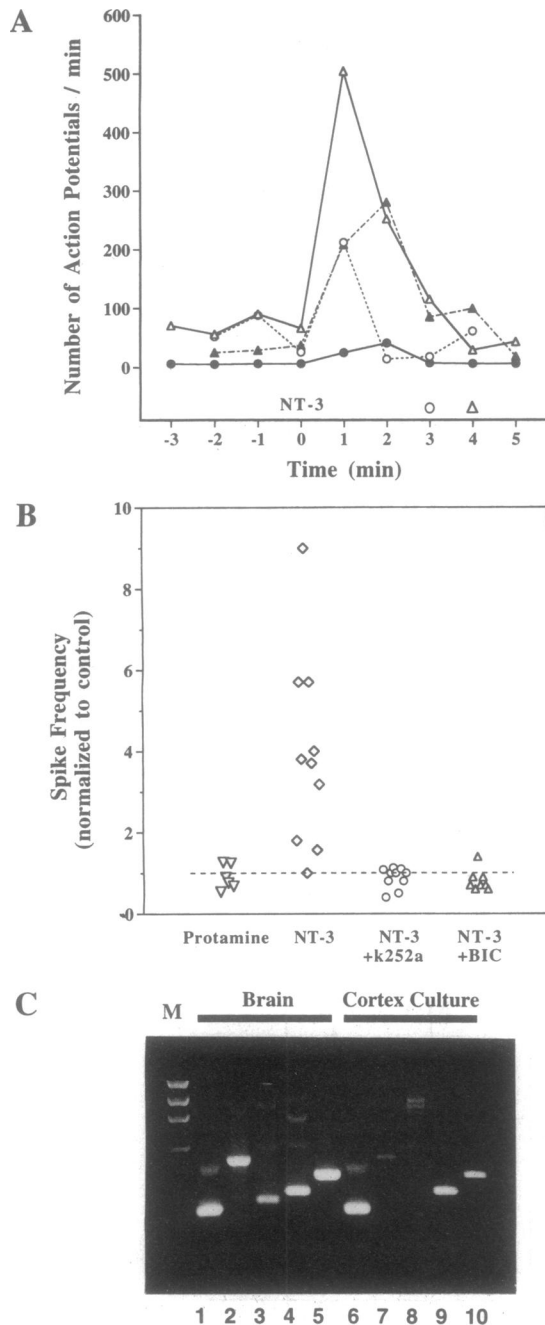
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FIG. 2. (*A*) Time course of increase in spike frequency induced by NT-3. Data from four different neurons are shown. NT-3 was perfused into the culture dish at time zero. In two cases (o and △), NT-3 was washed out, as indicated by corresponding symbols on the *x* axis. In two other cases (● and ▲), NT-3 was continuously present after time zero. Notice that these neurons exhibit a transient increase of spike frequency. (*B*) Changes in spike frequency under different conditions. Each point represents the ratio of the mean spike frequencies of the neurons treated with and without specific drugs indicated. The broken line represents the control level before drug treatment. The NT-3-induced increase in spike frequency is statistically significant (*t* test, $P < 0.005$). All drug solutions were made from bubbled Ringer solution and were applied in the bath. NT-3 and protamine were 5×10^{-10} M, k252a was 0.2 μM, and bicuculine (BIC) was 10 μM. In k252a experiments, cells were treated with the reagent for 1 hr before recording. (*C*) PCR detection of GAPDH (control), low-affinity NGF receptor (p75), and trk transcripts in total brain RNA (lanes 1–5) and RNA from cultures of cortical neurons (lanes 6–10). M, marker ϕX174 DNA, *Hae* III digest (in base pairs, from the top: 1353, 1078, 872, 603). Lanes 1 and 6, GAPDH (227 bp); lanes 2 and 7, p75 (501 bp); lanes 3 and 8, trkA (267 bp); lanes 4 and 9, trkB (309 bp); and lanes 5 and 10, trkC (399 bp).

were specific and mediated by the trk family of receptor tyrosine kinases.

Approximately 70% of neurons responded to NT-3. This is consistent with previous reports that only a subpopulation of cortical neurons expresses trk receptor tyrosine kinases (8–11, 31, 32). NT-3 is able to bind and activate all three trk receptor kinases (3, 4). To determine which trk receptor kinases are expressed in our cortical cultures, RNA from these cultures was reverse-transcribed and then amplified in a PCR using specific primers for different neurotrophin receptors. Similar to those in the whole brain, trkB, trkC, and low-affinity NGF receptor (p75) mRNAs were quite abundant in the cultured cortical neurons (Fig. 2*C*). A very low level of trkA mRNA was detected. Thus, NT-3 may regulate cortical neuronal activity through interaction of trkB and/or trkC receptors.

NT-3 did not have any noticeable effect on either resting membrane potentials or input resistance of the cortical neurons when measured in the presence of tetrodotoxin (TTX, a Na$^+$ channel blocker, 1 μM). These results suggest that the resting membrane conductance was not affected, and the effect of neurotrophins required action potential-driven synaptic activities. To determine the specific transmitter system(s) affected by NT-3, we first isolated the excitatory events by blocking the inhibitory synaptic transmission with the GABA$_A$ antagonist BIC (10 μM). Although BIC treatment increased activity in cortical neurons as expected (see also ref. 33), the spike frequency did not saturate because these neurons could generate higher frequency of action potentials by additional depolarization. If NT-3 acts through a mechanism independent of the GABA system, one would predict that inhibition of GABA activity should not prevent the increase of activity induced by NT-3, as long as BIC does not saturate the spike frequency. We found that in the presence of BIC, NT-3 no longer induced a further increase in the spike frequency (Fig. 2*B*, NT-3 + BIC: average ratio = 0.81 ± 0.09, mean ± SEM, $n = 8$). Thus, GABAergic synaptic events appear to be necessary for NT-3 effects. Since the increase in spike frequency induced by BIC was lower than that by NT-3 (20.4 vs. 156 spikes per min), there may be other mechanism(s) involved. Isolation of the inhibitory events by glutamate antagonists turned out to be difficult because blocking excitatory transmission also prevented spontaneous GABA-mediated events, probably by depriving excitatory inputs to these cells.

To directly test the role of NT-3 on synaptic transmission, we examined whether NT-3 can regulate the evoked synaptic transmission by obtaining recordings from pairs of synaptically connected neurons. Presynaptic neurons were stimulated at a low frequency (0.1 Hz) by a step depolarization to elicit a single action potential. EPSCs and IPSCs were recorded under voltage-clamped mode, after identifying each synaptic connection with either GABA or glutamate antagonists, respectively (10 μM BIC for GABA$_A$ receptor, 50 μM APV for NMDA receptor, and 100 μM DNQX for non-NMDA receptors). The inhibitory currents reversed between −40 and −50 mV (34), and they were GABAergic since they could be reversibly blocked by BIC ($n = 5$). In 10 of 15 pairs of neurons with an inhibitory connection, NT-3 significantly decreased the IPSC amplitude and maximal slope without affecting the decay time (*t* test, $P < 0.001$ for each pair). Statistical analysis of the pooled 15 GABA synapses showed an average of approximately 25% decrease for both peak amplitude and maximal slope, while the decay time of the GABA currents was not changed (Table 1). Examples of the neurotrophin-induced changes in IPSC amplitude and maximal slope are shown in Fig. 3 *A* and *B*, respectively. Again, NT-3 elicited a rapid and reversible reduction of the GABA-mediated synaptic currents. But unlike the NT-3 effects on spike frequency, the inhibition of IPSC persisted as long as

Table 1.   Effects of NT-3 on evoked synaptic currents

	Effect, % of control			
Current	Peak amplitude, pA	Maximal slope, pA/msec	Decay time, msec	n
GABA	76 ± 15*	75 ± 19*	101 ± 6	15
Glutamate	91 ± 8	93 ± 9	113 ± 5	10

The evoked synaptic currents were recorded as described in the legend of Fig. 3. Data were collected as paired measurements for the same cell over similar duration before and during the NT-3 treatment. The peak amplitudes and maximal slopes were obtained with the P-clamp analysis program. Decay phase of postsynaptic currents was fitted by a single exponential curve, and decay time was defined as the time needed for current amplitude to drop to $1/e$ of the peak value. The ratio between the mean value after NT-3 treatment and the mean control value was calculated for each synapse before averaging, and mean ± SD are presented. *, $P < 0.001$ (t test) compared with 100% for control.

NT-3 was in the medium. Fig. 3C shows 16 superimposed consecutive IPSC traces from another pair of neurons. The effects of NT-3 on IPSCs were also blocked by k252a (data not shown). Similar experiments were performed for excitatory synapses, and the excitatory currents can be blocked by a cocktail of APV and DNQX and reversed at 0 mV. It was very difficult to obtain pure evoked NMDA currents, because the currents are very small in these developing neurons. We therefore used the term "glutamate currents" to include both NMDA and non-NMDA currents. In contrast to its effects on IPSCs, NT-3 did not affect either the peak amplitude or the maximal slope of glutamate-mediated EPSCs (Table 1). Among 10 excitatory synapses, 6 exhibited no significant change, 1 had a slight increase, while 3 showed a decrease. Since NT-3 did not have an appreciable effect in the presence of BIC (Fig. 2B), glutamate-mediated currents did not seem

to be a major contributing factor for the increase of spike frequency. Thus, NT-3 induced potentiation of neuronal activity can be explained, at least in part, by its reduction of inhibitory synaptic transmission mediated by GABA$_A$ receptor.

DISCUSSION

We have examined the acute effects of neurotrophins on the neurons of the CNS, using whole-cell patch-clamp recording methods. Our results indicated that NT-3 rapidly increased the frequency of neuronal impulse activity in developing cortical neurons. We have also found that the potentiation of neuronal activity was achieved, at least in part, by inhibiting GABA-mediated synaptic transmission. These findings may be relevant to activity-dependent synaptic development and modulation.

The rapid and transient regulation of neuronal activity and synaptic activity reveals another mode of neurotrophin action. Traditionally, neurotrophins are viewed as slowly acting, long-lasting, target-derived differentiation factors (35, 36). Although the signal transduction of the neurotrophins (including tyrosine phosphorylation, c-fos induction, etc) takes place in seconds or minutes, the functional events, such as regulation of neuronal survival, neurite outgrowth, and phenotypic differentiation, usually takes hours, sometimes days. Our present study indicates that the frequency of action potentials and synaptic activities of cortical neurons were changed very rapidly by neurotrophin treatment. More interestingly, the neurotrophin effects on neuronal activity were transient. It is therefore possible to generalize the finding of rapid action of neurotrophins at developing neuromuscular junctions to CNS neurons (5) and to suggest an additional role of neurotrophins in the nervous system,
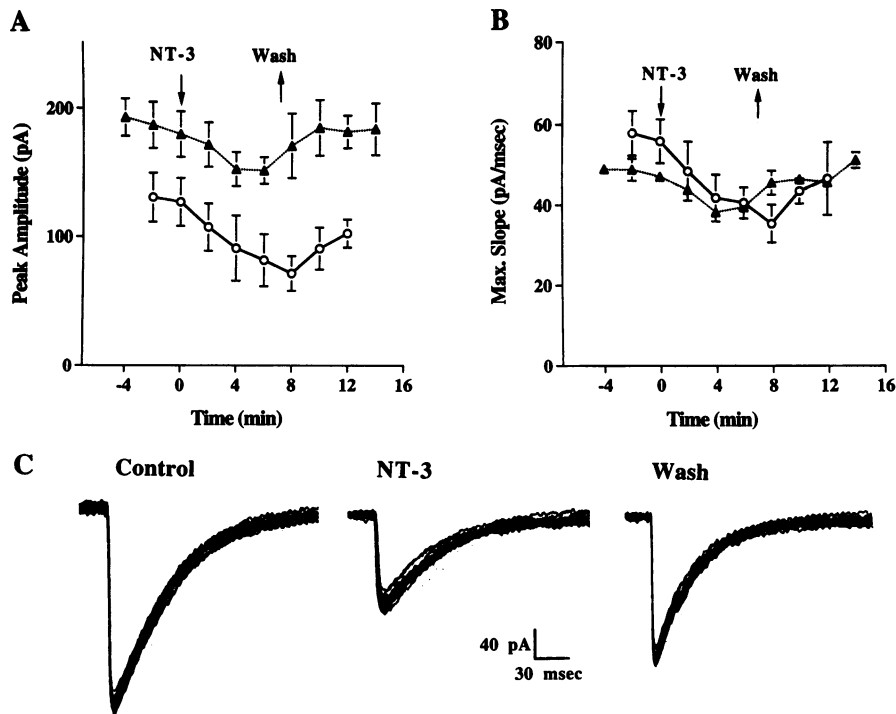

FIG. 3.   Effect of NT-3 on evoked IPSCs. (A) Time course of inhibition of the IPSC peak amplitude. The decrease of IPSC amplitude induced by NT-3 treatment was statistically significant ($P < 10^{-7}$, t test). Error bars indicate the SD. Each point represents an average of 12 IPSCs obtained by firing action potentials in the presynaptic neuron at 10-sec intervals. (B) Time course of inhibition of the IPSC maximal slope. The data points are mean ± SEM. The decrease of maximal slope was also statistically significant ($P < 0.0001$, t test). The same symbol used in A and B (▲ or O) represents the peak amplitude and maximal slope of the same cell. The data points before NT-3 application fluctuated but were not statistically different. (C) Samples of IPSCs in control (Left), NT-3 treatment (Center), and wash (Right). Sixteen superimposed consecutive current traces of a different paired neurons from a 14-day culture are shown. NT-3 reversibly reduced the amplitude of IPSCs. $V_{hold} = -60$ mV. The currents are seen as inward, since the reversal potential (V_{rev}) was around -45 mV.
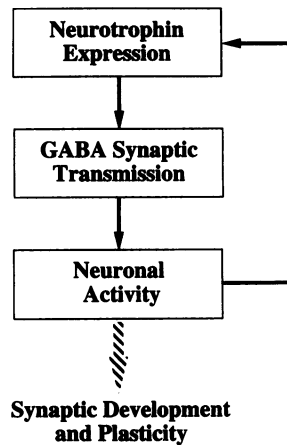
FIG. 4. Diagram of the transient and reciprocal interaction between neuronal activity and neurotrophins, and its implications.

namely to serve as neuromodulators, regulating synaptic efficacy and patterns of neuronal activity.

The neuromodulatory role of neurotrophins has been implicated by the activity-dependent expression of these factors in the CNS neurons. Neuronal depolarization and excitatory transmitters elicit a rapid increase, while inhibitory transmitters cause a decrease in the levels of neurotrophin mRNAs in hippocampal neuronal cultures (17–19). The neurotrophin gene expression is also dramatically enhanced in hippocampus and neocortex a few hours after experimentally induced seizures (13–15). We now provide evidence that neuronal impulse activity and synaptic transmission in cultured cortical neurons can be regulated by NT-3. Our present observations have led to a particularly attractive hypothesis that there may exist a positive feedback loop (Fig. 4); impulse activity enhances neurotrophin expression, which in turn potentiates neuronal activity and synaptic efficacy.

The reciprocal interaction between neurotrophins and neuronal activity may be significant in the activity-dependent synaptic modulation during development and in the adult. Indeed, sensory inputs have been shown to regulate neurotrophin gene expression in the neocortex (16). Stimulus paradigms inducing long-term potentiation (LTP) in the hippocampus also evoke significant increases in the levels of NT-3 mRNA (ref. 21, but see ref. 22). In the present study, we have demonstrated that NT-3 can inhibit GABA$_A$-mediated transmission. We have also shown that the potentiation of neuronal activity can be attributable, at least in part, to the reduction of GABAergic inhibitory activity. Since GABA is a major inhibitory transmitter in the CNS, our finding of neurotrophin modulation of GABAergic transmission suggests previously unsuspected roles and mechanisms for neurotrophins in CNS functioning and plasticity (Fig. 4). For example, inhibition of GABA activity has been shown to play an important role in eliciting LTP in the neocortex (37) and in cortical plasticity (38). Our results here raise the interesting and testable hypothesis of neurotrophin involvement in cortical synaptic plasticity.

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