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Blocking the NGF-TrkA Interaction Rescues the Developmental Loss of LTP in the Rat Visual Cortex: Role of the Cholinergic System

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Summary

Although nerve growth factor (NGF) is a crucial factor in the activity-dependent development and plasticity of visual cortex, its role in synaptic efficacy changes is largely undefined. We demonstrate that the maintenance phase of long-term potentiation (LTP) is blocked by local application of exogenous NGF in rat visual cortex at an early stage of postnatal development. Long-term depression (LTD) and bidirectional plasticity are unaffected. At later postnatal ages, blockade of either endogenous NGF by immunoadhesin (TrkA-IgG) or TrkA receptors by monoclonal antibody rescues LTP. Muscarinic receptor activation/inhibition suggests that LTP dependence on NGF is mediated by the cholinergic system. These results indicate that NGF regulates synaptic strength in well-characterized cortical circuitries.

Introduction

Neurotrophins of the nerve growth factor family (NGF, BDNF, NT-3, NT-4/5) are small, related proteins that bind to specific receptors (Trk and P75; reviewed by Chao and Hempstead, 1995) and exert a trophic action on specific subgroups of neurons both in the PNS and the CNS. The prototype of these factors is nerve growth factor (NGF), discovered more than 45 years ago by Levi-Montalcini (reviewed by Levi-Montalcini, 1987).

Beyond its classic role in neuronal survival, neurotrophins regulate important aspects of neuronal development, such as phenotype differentiation and/or maintenance (reviewed by Lewin and Barde, 1996). An increasing body of evidence indicates that neurotrophins control neuronal plasticity during development. For example, an exogenous supply of NGF is able to prevent the effects induced by manipulations of visual experience, such as monocular deprivation and artificial strabismus, performed during a restricted time window (critical period) of postnatal development (Domenici et al., 1991, 1992, 1993; Maffei et al., 1992). Blockade of endogenous NGF by antibodies prevents the normal activity-dependent development of the geniculocortical system (Berardi et al., 1994) and delays the end of the critical period for monocular deprivation (Domenici et al., 1994). Furthermore, brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) can modulate some aspects of visual cortex development (Cabelli et al., 1995; McAllister et al., 1995, 1996) and developmental cortical plasticity (Riddle et al., 1996). Neurotrophins and their receptors are expressed at the level of visual cortex. Interestingly, the BDNF mRNA endogenous level is regulated by light; regulation of NGF is more complex and is possibly under the control of several factors, including light (Schoups et al., 1995). All of these experiments point to the fact that neurotrophins are molecules expressed under the control of neuronal activity which act by modulating developmental cortical plasticity and postnatal development in mammalian visual cortex (reviewed by Thoenen, 1995; Bonhoeffer, 1996; Cellerino and Maffei, 1996).

The mechanisms through which these neurotrophins are involved in cortical development and/or plasticity are not yet understood. Neurotrophins could act through intracellular signals, converting neuronal activity into long lasting changes in synaptic strength (reviewed by Katz and Shatz, 1996). Two forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD), are expressed in visual cortex and are NMDA dependent (reviewed by Kirkwood and Bear, 1995). More recently, a novel form of synaptic plasticity, called bidirectional plasticity, has been described in hippocampus by Dudek and Bear (1993) and in developing visual cortex by Sermasi et al. (1999a). All of these forms of long-term changes in synaptic efficacy are present at the level of visual cortex soon after eye opening (Kirkwood and Bear, 1995; Kirkwood et al., 1995; Sermasi et al., 1999a, 1999b), i.e., at an early stage of postnatal development. LTP, LTD, and bidirectional plasticity are expressed under the control of visual experience, which represents the instructive message for the postnatal development and plasticity of visual cortex.

In spite of a large amount of data on the effects of NGF on the activity-dependent postnatal development of visual cortex, the question of whether NGF is able to modulate the different forms of synaptic efficacy changes is still unanswered. The principal aims of the present work are to investigate (1) the effects induced by an acute NGF supply or NGF blockade on the expression of LTP, LTD, and bidirectional plasticity at different stages of postnatal development of visual cortex and (2) whether NGF effects on synaptic plasticity are mediated by the cholinergic system and, in particular, by basal forebrain (BF) neurons that project to the cortex and express NGF receptors (Seiler and Schwab, 1984; Steininger et al., 1993; Sobreviela et al., 1994; Li et al., 1995). To these aims, extracellular field potentials (fEPSPs) were recorded in slices containing the primary visual cortex. Slices were examined at different postnatal ages: P16–P18 (1–3 days after eye opening), the beginning of the critical period for monocular deprivation in rat visual cortex (Fagiolini et al., 1994) and the age(s) at which LTP is maximally expressed (Kirkwood et al.,

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1995), and P30–P35, the age(s) at which the critical period for monocular deprivation is almost over. The effects of NGF-TrkA interaction were investigated by performing electrophysiological recordings combined with local delivery of substances through the recording pipette. Blockade of NGF was achieved by using a specific immunoadhesin, and inhibition of TrkA was obtained by using a neutralizing monoclonal antibody.

Results

Immunoadhesins and Antibody Characterization

To sequester endogenous NGF, we used a soluble form of TrkA receptor engineered as an immunoadhesin, a chimeric molecule combining the binding site region of the TrkA receptor with the Fc region of an immunoglobulin (IgG) (reviewed by Ashkenazi and Chamow, 1997). A new form of immunoadhesin was used in the present study (TrkA immunoadhesin, TrkA-IgG, and TrkB immunoadhesin, TrkB-IgG), in which the binding-site regions came from human TrkA and TrkB receptors, respectively, and the Fc region from camel IgG_2, composed of a long, proline-rich hinge (35 amino acids) followed by the C_H2 and C_H3 domains (Hamers-Casterman et al., 1993). This natural form of camel heavy chain lacks the C_H1 domain and therefore can be secreted in the absence of a light chain, avoiding the need to artificially engineer the deletion of the C_H1 domain, as has to be done in conventional immunoadhesins (reviewed by Ashkenazi and Chamow, 1997).

The specificity of immunoadhesins for different neurotrophins was assessed by enzyme-linked immunosorbent assays (ELISAs): applying three different concentrations of NGF and BDNF (50, 100, and 500 ng/ml) to solid, phase-coupled TrkA- and TrkB-IgG (Figures 1A and 1B). TrkA-IgG showed high specificity for NGF, while TrkB-IgG did not recognize this neurotrophin. Only at the highest concentration of BDNF was there a very limited binding of TrkA-IgG.

In this paper, we also made use of a newly derived monoclonal antibody recognizing the extracellular portion of rat and human TrkA receptors. The MNAC13 anti-TrkA monoclonal antibody (αTrkA) is an antagonistic antibody that fully inhibits the binding of ^{125}I-NGF to TrkA receptor–expressing cells (Cattaneo et al., 1999). αTrkA was derived from Balb/C mice immunized with Balb/C 3T3 cells expressing human TrkA receptor and was isolated on the basis of its ability to inhibit the binding of NGF to TrkA. The specificity of purified αTrkA was confirmed by ELISA (Figures 1C and 1D): applying two different concentrations of αTrkA (1, 5 μg/ml) to plates coated with two different concentrations of either TrkA-IgG or TrkB-IgG (0.1, 1 μg/ml). The antibody did not bind TrkB-IgG, while it recognized specifically TrkA-IgG.

Bioassays on PC12 cells (Greene and Tischler, 1976) were performed in order to verify the action of immunoadhesins, αTrkA, and heat-inactivated NGF (Den.NGF) used in electrophysiological experiments. PC12 cells cultured for 1 week in the absence of NGF did not differentiate (Figure 2A; percentage of differentiated cells [PDC] = 0%, 3 fields). Addition of NGF (20 ng/ml) to the incubation medium for 1 week promoted neuronal differentiation of PC12 cells (Figure 2B; PDC = 67.5% ± 5.2%, 6 fields). TrkA-IgG was capable of blocking the physiological action of exogenous NGF by inhibiting the NGF-induced differentiation (Figure 2C; PDC = 0%, 3 fields), while in the presence of TrkB-IgG, cells differentiated (Figure 2D; PDC = 61.5% ± 3.4%, 6 fields). Addition of TrkA-IgG alone did not result in PC12 cell differentiation (data not shown; PDC = 0%, 4 fields). We also tested the ability of αTrkA to block the NGF-TrkA activation. PC12 cells treated with NGF and αTrkA (Figure 2E; PDC = 0%, 4 fields) did not differentiate. Moreover, incubation of PC12 cells with αTrkA in the absence of NGF did not result in their differentiation (data not shown; PDC = 0%, 6 fields), proving that the antibody does not have agonistic activity due to receptor cross-linking. In contrast, PC12 cells incubated with NGF and 9E10 αMyc, a monoclonal antibody against an intracellular epitope used as negative control, differentiated normally (Figure 2F; PDC = 63.5% ± 4.8%, 5 fields). Finally, PC12 cells treated with Den.NGF did not show neuronal differentiation (Figure 2G; PDC = 0%, 4 fields). Den.NGF was recognized by NGF polyclonal antibody (αNGF) in ELISA, indicating a preservation of its immunological properties. On the contrary, Den.NGF failed to bind TrkA-IgG, confirming the loss of its function (Figure 2H).

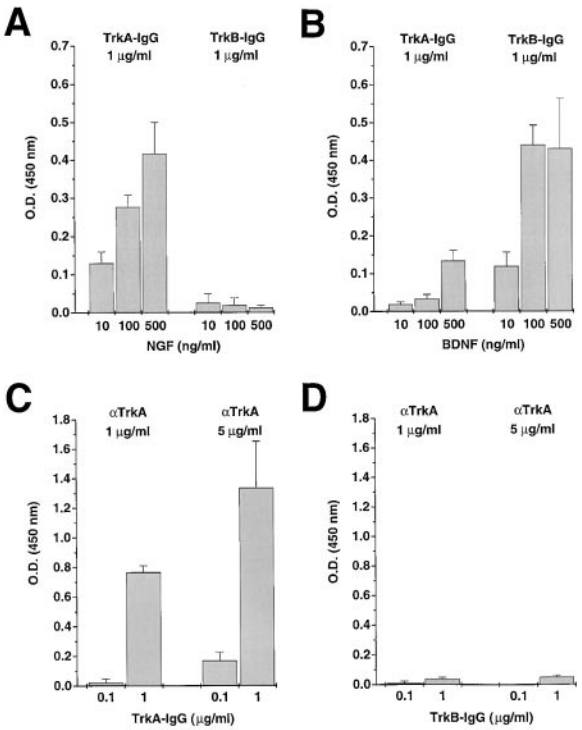

Figure 1. Specificity of Neurotrophins and αTrkA for Immunoadhesins

(A and B) Different concentrations of NGF (A) and BDNF (B) were added both to TrkA-IgG and TrkB-IgG adsorbed on plates. Each neurotrophin shows high specificity for its own corresponding immunoadhesin.

(C and D) Two different concentrations of αTrkA were added to TrkA-IgG (C) and TrkB-IgG (D) adsorbed on plates. αTrkA recognizes TrkA-IgG with high specificity and in a dose-dependent manner. Moreover, there seems to be a threshold under which recognition is not appreciable.

Thus, TrkA-IgG and αTrkA specifically block the biological activity of NGF-TrkA interaction.

Diffusion of Locally Released Compounds

To assess the maximal spreading of delivered compounds, we locally released biocytin through the recording pipette. It is known that neurons are able to take up biocytin applied extracellularly and can convey it via anterograde and retrograde transport; thus, biocytin represents a useful tool for labeling individual neurons and for tracing neural connections (reviewed by McDonald, 1992). We applied biocytin (either 0.5% in 0.15 M NaCl or 0.1% in 1 M NaCl) to slices of rat visual cortex through the recording pipette, and we mimicked an electrophysiological experiment in order to assess the extent of substance diffusion from the recording site. The intensity of labeling was proportional to the concentration of biocytin loaded into the pipette (Figures 3A–3D). At the lowest concentration (0.1%; Figures 3C and 3D), just a few neurons were labeled, thus supporting the idea of a spatially restricted action of compounds delivered through the recording pipette.

Exogenous Supply of NGF Affects LTP

At P16–P18, stable LTP was reliably elicited by high-frequency stimulation (HFS) (Figures 4A, 7A, and 7B; after 50 min, mean fEPSP = 137% ± 11%, n = 8, 8 rats). In the presence of NGF (100 ng/ml) in the recording pipette, potentiation was blocked (Figure 4A); in fact, 20–25 min after tetanic stimulation, fEPSP amplitude was not significantly different from the baseline condition (Figures 4A, 7A, and 7B; after 25 min, mean fEPSP = 108% ± 9.2%, after 50 min, mean fEPSP = 91.4% ± 7.9%, n = 10, 8 rats, $p \leq 0.001$). Local supply of NGF did not change the basic properties of field potentials, namely, their amplitude, shape, and dependence on stimulation rate. To control for the specificity of the inhibition, by NGF, of potentiation, slices were treated with locally delivered, heat-inactivated NGF. Results reported in Figures 4A, 7A, and 7B clearly show that LTP was not altered by local delivery of Den.NGF (100 ng/ml; after 50 min, mean fEPSP = 131% ± 7%, n = 7, 5 rats). Den.NGF was tested on PC12 cells for potential residual activity (Figure 2G) immediately before slice application.

In a further set of experiments, we showed that NGF effect was restricted to LTP and did not extend to other forms of synaptic plasticity. Stable LTD was induced by low-frequency stimulation (LFS) of white matter (WM) in all control slices at P16–P18. Data reported in Figure 4B indicate that the amplitude of LTD was not significantly different in control and NGF- (100 ng/ml) treated slices (40 min after LFS, mean fEPSP = 66.3% ± 3.7%, n = 11, 9 rats and 65.1% ± 4.9%, n = 6, 4 rats, respectively).

In a previous paper (Sermasi et al., 1999a), it was reported that it is possible to potentiate previously depressed synapses at an early stage of postnatal development (P16–P18). To test if NGF would affect this form of synaptic plasticity, we compared the amplitude of potentiation after tetanic stimulation of previously depressed synapses. From Figure 4B, it appears that HFS of previously depressed synapses reliably elicited potentiation in both NGF-treated and untreated slices without significant differences.

LTP Dependence on Endogenous NGF Level

The evidence that exogenous NGF supply was able to block LTP at P16–P18 prompted us to investigate the role of endogenous NGF on this form of synaptic plasticity. To this aim, we utilized TrkA-IgG at the concentration (1 μg/ml) known to prevent NGF-dependent differentiation of PC12 cells. This TrkA-IgG concentration is several orders of magnitude greater than the reported cortical endogenous NGF level (Whittemore et al., 1986; Larkfors and Ebendal, 1987). With TrkA-IgG in the pipette filling solution, we observed spontaneous bursts of activity (afterdischarge) during HFS protocol, a clear index of neuronal hyperexcitability. Despite this effect, LTP was not significantly affected in TrkA-IgG-treated slices (Figures 4C, 7A, and 7B; after 50 min, mean fEPSP = 144% ± 18%, n = 8, 6 rats).

Having first established that blockade of endogenous NGF at P16–P18 does not affect LTP, we examined the potential role of endogenous NGF at P30–P35, when LTP is no longer inducible. Blockade of endogenous NGF by TrkA-IgG at a concentration of 1 μg/ml was able to rescue LTP following HFS protocol (Figures 5A, 7A, and 7B; after 50 min, mean fEPSP = 124% ± 9%, n = 10, 6 rats, $p \leq 0.001$), as compared with control slices (Figures 5A, 7A, and 7B; after 50 min, mean fEPSP = 96.3% ± 3.3%, n = 8, 8 rats). Since TrkA-IgG, at high concentration, showed some very limited cross-reactivity with BDNF (Figure 1B), we asked whether reappearance of LTP was due to blockade of other related neurotrophins besides NGF. To answer this question, we made use of TrkB-IgG, an immunoadhesin sequestering endogenous BDNF and NT-4. In Figure 5A, we report that locally delivered TrkB-IgG (1 μg/ml) was not able to reinduce LTP at P30–P35 (Figures 5A, 7A, and 7B; after 50 min, mean fEPSP = 101% ± 3%, n = 8, 7 rats).

In a second group of experiments, we examined the role of TrkA on LTP at P30–P35. Monoclonal antibody–blocking TrkA receptors were included in the pipette filling solution. αTrkA (5 μg/ml) induced the reappearance of LTP (Figures 5B, 7A, and 7B; after 50 min, mean fEPSP = 132% ± 10%, n = 8, 6 rats, $p \leq 0.001$), in analogy to what we found using TrkA-IgG.

Interaction between the BF Cholinergic System and NGF

A large body of evidence indicates that TrkA receptor protein is present in the visual cortex, maximally expressed on BF cholinergic terminals (Steininger et al., 1993; Sobreviela et al., 1994; Li et al., 1995; Molnar et al., 1998; Pizzorusso et al., 1999). The possible involvement of the cholinergic system in NGF dependence of LTP was examined by performing experiments both at P16–P18 and P30–P35. In particular, we made use of acute and local delivery of the muscarinic receptors' agonist and antagonist.

By using pharmacological tools, we found that blockade of muscarinic receptors by atropine (100 μM), delivered through the recording pipette, diminishes LTP (Figures 6A, 7A, and 7B; after 50 min, mean fEPSP = 94.8% ± 7.8%, n = 7, 4 rats, $p \leq 0.001$). Indeed, HFS in the presence of atropine induced a transient potentiation lasting only about 25–30 min. By contrast, muscarine (100 μM) administration permitted a normal LTP

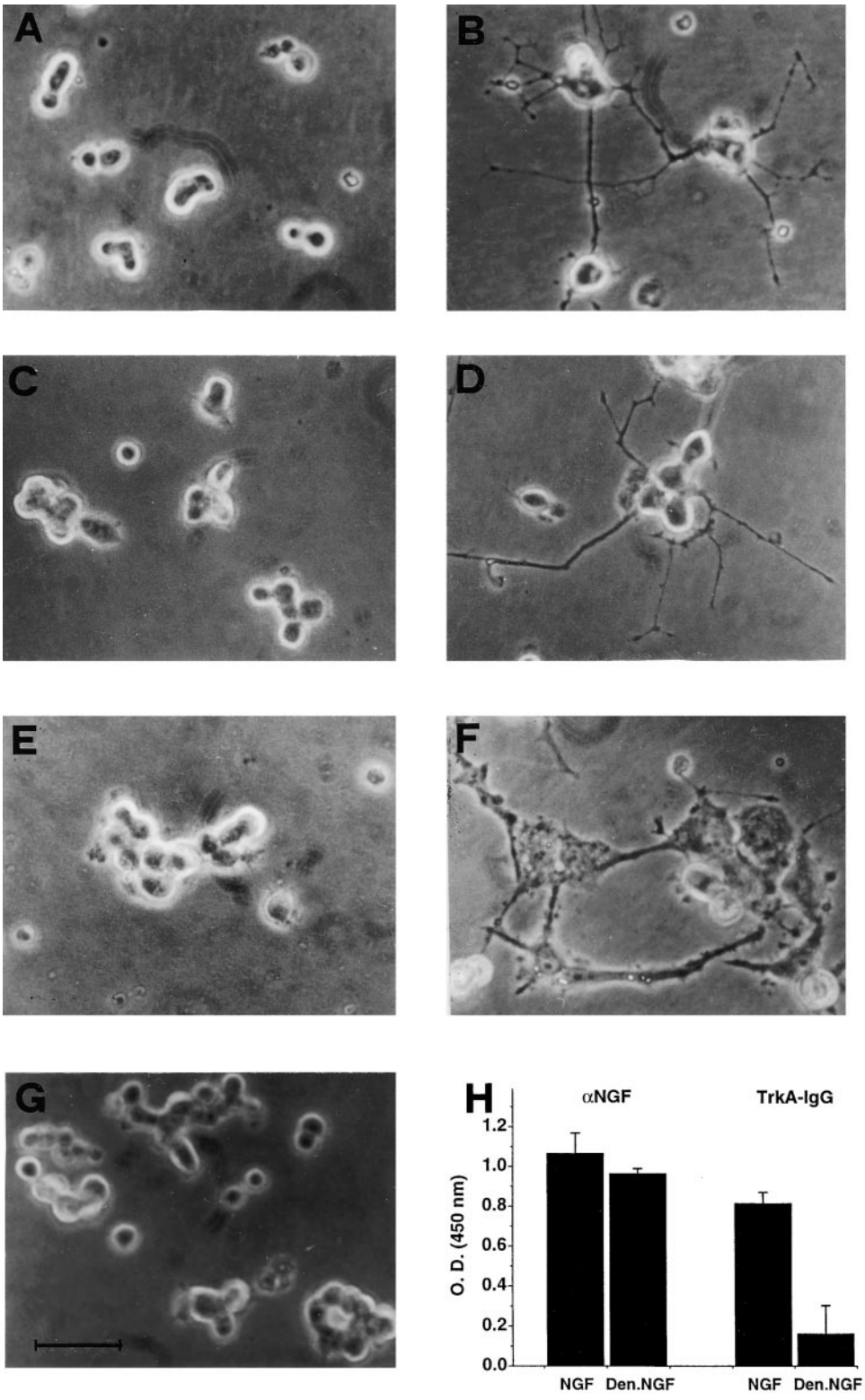

Figure 2. Action of TrkA-IgG and αTrkA on PC12 Cells

(A–G) PC12 cells were grown in 1% HS and in the presence of NGF and different immunoadhesins and antibodies: TrkA-IgG, TrkB-IgG, αTrkA, and 9E10 αMyc.

(A) Undifferentiated PC12 cells grown in normal medium (1% HS) for 1 week.

(B) Differentiated PC12 cells grown in the presence of NGF (20 ng/ml).

(C) Undifferentiated PC12 cells grown in normal medium and NGF in the presence of TrkA-IgG (3 μg/ml).

(D) Differentiated PC12 cells grown in normal medium and NGF in the presence of TrkB-IgG (3 μg/ml).

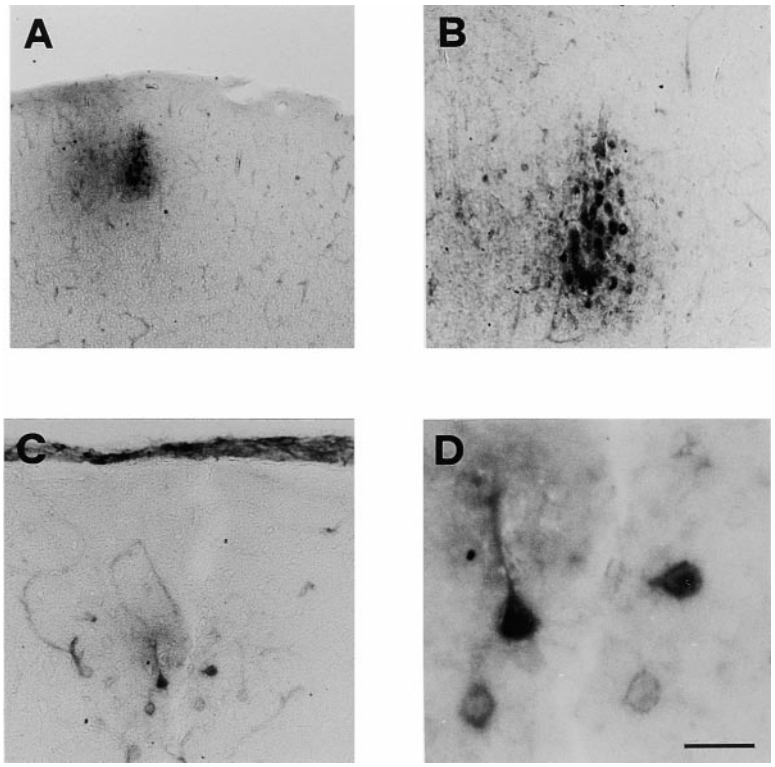

Figure 3. Biocytin Labels Neurons Surrounding Recording Pipette

(A and B) Labeling of neurons by uptake of 0.5% biocytin in 0.15 M NaCl solution.
(C and D) Labeling of neurons by uptake of 0.1% biocytin in 1 M NaCl solution.
Scale bar in (D), 400 μm (A); 100 μm (B); 80 μm (C); and 25 μm (D).

(time course not shown; Figures 7A and 7B; after 50 min, mean fEPSP = 122% ± 4%, n = 6, 3 rats). Thus, blockade of muscarinic receptors mimicked the effect of NGF on LTP. At P30–P35, HFS in the presence of muscarine (100 μM) reinduced LTP, whose amplitude was comparable to that obtained by TrkA-IgG addition (Figures 6B, 7A, and 7B; after 50 min, mean fEPSP = 138% ± 9%, n = 7, 5 rats, $p \leq 0.001$). Particularly interesting is that LTP could not be reinduced when atropine (100 μM) was locally supplied together with TrkA-IgG (1 μg/ml; Figures 6B, 7A, and 7B; after 50 min, mean fEPSP = 104% ± 8%, n = 8, 5 rats).

Discussion

In the present paper, we show that LTP expression, but not other forms of synaptic plasticity, in rat visual cortex during postnatal development depends on the local level of NGF. In addition, our results strongly suggest that NGF interacts with the cholinergic system in modulating LTP.

Local Delivery of Compounds

The unusual feature of the present work is the delivery of drugs through the recording pipette filling solution. This novel approach allowed us to avoid a general, undiscriminated stimulation of the entire cortical network and to circumscribe the action of substances to a small area surrounding the pipette tip. Reported effects of drugs in previous works (Brocher et al., 1992; Tancredi et al., 1993; Sokolov and Kleschevnikov, 1995) led us to estimate a 10- to 100-fold dilution, close to pipette, when substances dissolved in the pipette filling solution were delivered into the slice. In accordance with this estimation, as a general rule we have employed concentrations 10-fold higher than those found to be effective when the same compounds were added to perfusion medium.

In an attempt to estimate the range of diffusion of substances coming out of the pipette tip, we made use of different biocytin concentrations locally supplied through the recording pipette. The lower molecular weight of biocytin presumably causes a faster diffusion as compared with proteins, such as NGF and immunoadhesins. However, this is probably compensated for by a less effective uptake due to the interaction of biocytin with low-affinity amino acid receptors. The reported results show that at the lowest concentration used (which is still, at least, 100-fold higher than the concentration of proteins/drugs employed in our experiments), just a few neurons are labeled, thus supporting the idea of a spatially restricted action of substances delivered following this method.

NGF Modulates LTP during Postnatal Development

Different forms of long-term changes in synaptic efficacy are differentially regulated during postnatal development of rat visual cortex. LTP is maximally expressed

(E) Undifferentiated PC12 cells grown in normal medium and NGF in the presence of αTrkA (4 μg/ml).
(F) Differentiated PC12 cells grown in normal medium and NGF in the presence of αMyc (4 μg/ml).
(G) Denatured NGF (20 ng/ml) was unable to differentiate PC12 cells.
(H) Two-site ELISA shows that denatured NGF is unable to bind to TrkA-IgG, but it retains its immunogenicity by binding to αNGF.
Scale bar in (G), 50 μm (A–G).

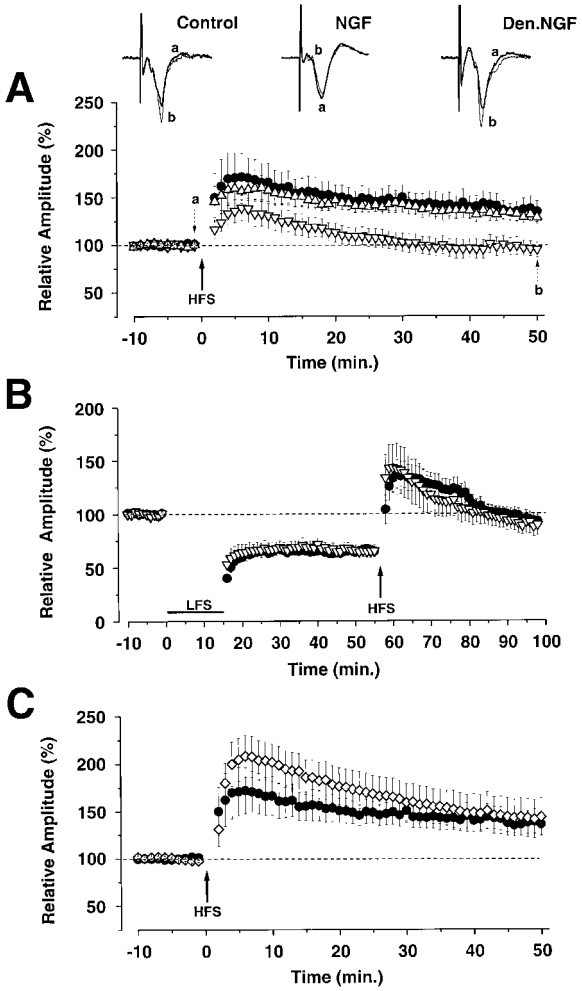

Figure 4. Effects of NGF and TrkA-IgG on Synaptic Plasticity at P16–P18

(A) Local application of NGF (100 ng/ml; open upside-down triangles) is able to block potentiation, as compared with control slices (closed circles). Heat-inactivated NGF (100 ng/ml; open triangles) is unable to prevent LTP expression. Insets depict representative field potentials at the tenth minute of baseline recording (Aa) and at the fiftieth minute after HFS protocol (Ab).

(B) Local administration of NGF (100 ng/ml; open upside-down triangles) does not affect either LTD or potentiation of previously depressed synapses, as compared with controls (closed circles).

(C) Local depletion of endogenous NGF by TrkA-IgG (1 μg/ml; open diamonds) administration does not significantly alter LTP, as compared with control slices (closed circles).

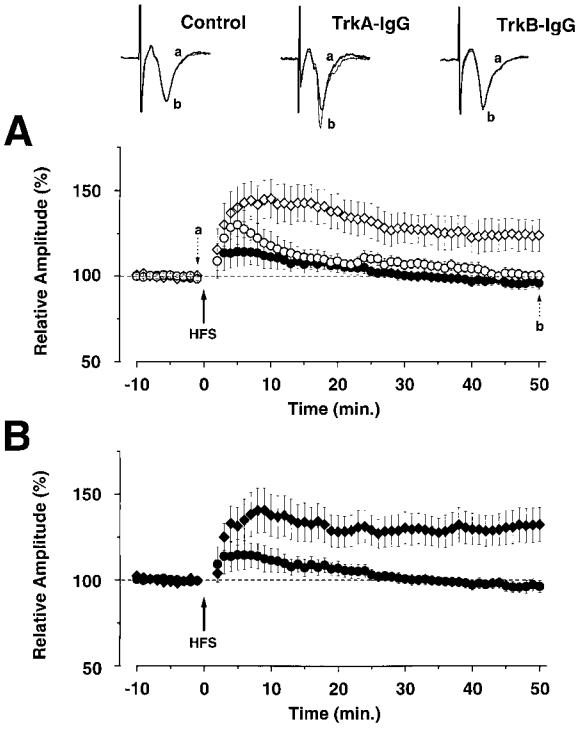

Figure 5. Effects of Endogenous NGF Action Blockade on Synaptic Plasticity at P30–P35

(A) Blockade of endogenous NGF by local TrkA-IgG (1 μg/ml; open diamonds) application rescues the ability of slices to be potentiated, as compared with controls (closed circles). Conversely, blockade of endogenous BDNF by TrkB-IgG (1 μg/ml; open circles) does not enable LTP to be reexpressed. Insets depict representative field potentials at the tenth minute of baseline recording (Aa) and at the fiftieth minute after HFS protocol (Ab).

(B) Blockade of TrkA receptors by local administration of αTrkA (5 μg/ml; closed diamonds) allows reexpression of LTP, as TrkA-IgG, in response to HFS protocol, as compared with controls (closed circles).

soon after eye opening, at P16–P18, and is progressively downregulated thereafter, completely disappearing after P30 (Kirkwood et al., 1995). Synapses are capable of reversing a given long-term change in synaptic efficacy (bidirectional plasticity) at P16–P18, but this property is lost 1 week later (Sermasi et al., 1999a). Finally, LTD is expressed throughout life, although its amplitude is reduced in adult animals (Sermasi et al., 1999b). In this study, we tried to identify possible factors modulating the expression of different forms of synaptic plasticity during postnatal development. Neurotrophins have been proposed as likely candidates, as they have been reported to influence activity-dependent development and plasticity of visual cortex (reviewed by Thoenen, 1995; Bonhoeffer, 1996). NGF has been shown to prevent the geniculocortical alterations induced by monocular deprivation performed during a restricted time window of postnatal development (critical period) (reviewed by Cellerino and Maffei, 1996).

We have found that at an early stage of postnatal development (P16–P18), a local supply of NGF in layer II/III affects LTP elicited by a tetanic stimulation of WM in the rat visual cortex. In particular, synaptic potentiation cannot be maintained longer than 20–25 min. The NGF effect on potentiation is specific, since control experiments with biologically inactive NGF leave LTP amplitude completely normal. Thus, NGF seems to play an inhibitory role in LTP elicited by WM tetanic stimulation. This NGF effect on synaptic plasticity is restricted to LTP. Indeed, neither LTD nor LTP of previously depressed synapses elicited by stimulation of WM are affected by NGF; it may well be that these two forms of synaptic plasticity rely on other neurotrophins and/or different factors and mechanisms (reviewed by McAllister et al., 1999). Therefore, we showed a specific and selective effect of NGF on LTP expression in a characterized circuitry of primary visual cortex.

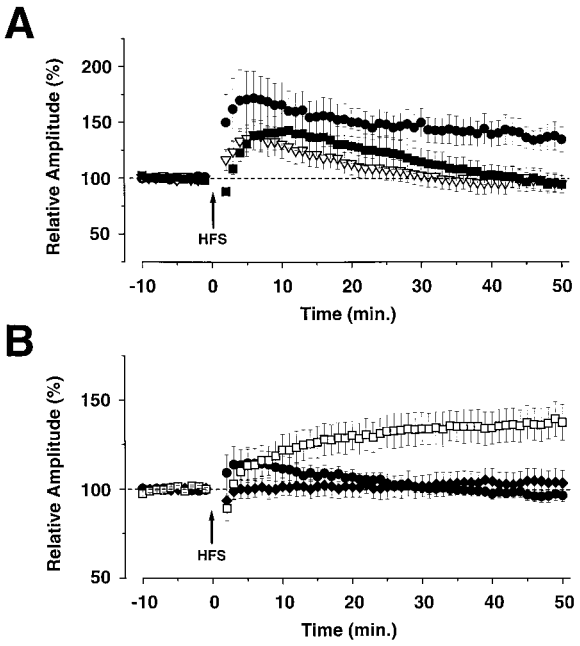

Figure 6. The Cholinergic System Is Involved in NGF Action

(A) At P16–P18, local application of the muscarinic receptor antagonist atropine (100 μM; closed squares) mimics the effect of NGF (open upside-down triangles) application by inhibiting potentiation, as compared with controls (closed circles).

(B) At P30–P35, activation of muscarinic receptors by muscarine (100 μM; open squares) leads to potentiation of field potential as, compared with controls (closed circles), in response to HFS protocol. On the contrary, the muscarinic receptor antagonist atropine (100 μM; closed diamonds) is able to prevent potentiation in TrkA-IgG-treated slices.

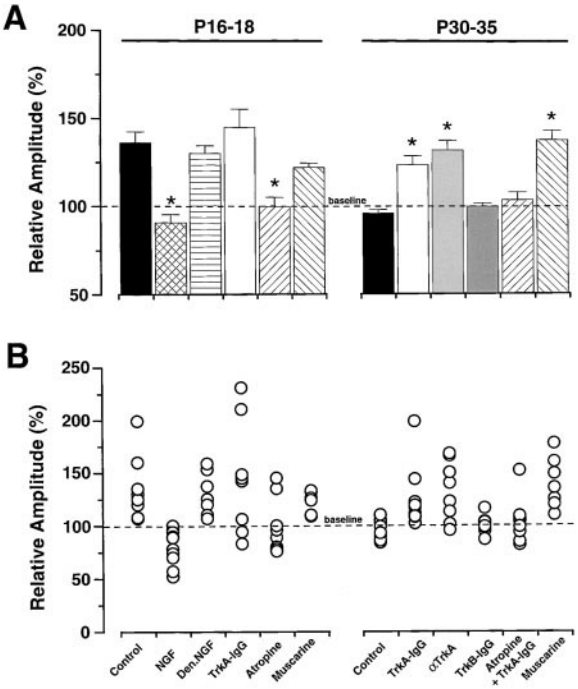

Figure 7. Summary of Results

(A) Columns represent the average of 3 min field potential amplitudes (centered on 50 min after HFS protocol), pooling together all n slices for each kind of treatment. Columns indicate mean relative amplitude + SEM, compared with baseline (asterisk, $p \leq 0.001$ by Rank Sum test).

(B) Representation of averaged field potential amplitudes of each single slice, sampled along 3 min (centered on 50 min after HFS protocol) and grouped for each kind of treatment. Each circle indicates mean relative amplitude, compared with baseline.

In a previous paper (Akaneya et al., 1997) dedicated to the role of neurotrophins in visual cortex synaptic plasticity, it was reported that exogenously supplied NGF is not able to induce modifications of LTP elicited by stimulation of layer IV. Possible discrepancies could depend upon experimental differences; indeed, in that study, NGF was dissolved in the perfusion medium, while in our experiments it was locally supplied. In addition, stimulation of layer IV induces activation of a different type of intracortical circuitry with respect to stimulation of WM (Kirkwood and Bear, 1994).

At P16–P18, blockade of endogenous NGF achieved by local supply of soluble forms of TrkA receptor (TrkA-IgG) induces spontaneous bursts of field potentials during tetanic stimulation but does not affect LTP expression. We suggest that the basal endogenous NGF level at this stage of postnatal development allows for maximal expression of LTP (Kirkwood et al., 1995). When NGF goes up under the action of physiological stimuli or because it is exogenously supplied, LTP amplitude is dramatically reduced. Endogenous NGF blockade does not change LTP amplitude, probably because at this time HFS of WM occludes further strengthening of the synaptic response.

In keeping with the hypothesis that LTP expression depends on NGF level, we have found that blocking endogenous NGF by local supply of TrkA-IgG induces the reappearance of LTP at an age when this form of synaptic plasticity is normally no longer expressed (P30–P35). In addition, we have demonstrated that this effect is mediated by TrkA receptors; indeed, blockade of TrkA receptors, by neutralizing αTrkA, produces the same effect as endogenous NGF blockade, i.e., reinduction of LTP expression following tetanic stimulation.

It is well known that during postnatal development, visual cortical neurons are influenced by manipulations of visual experience. A classic example is the loss of responsiveness to stimulation of an eye that has been deprived of vision. In early experiments, it was established that an exogenous supply of neurotrophins and, in particular, NGF prevents the physiological and morphological consequences of monocular deprivation during the critical period (Domenici et al., 1991, 1993; Maffei et al., 1992). More recent data indicate that NGF effects are mediated by TrkA; indeed, activation of TrkA by polyclonal antibodies is able to prevent the effects of monocular deprivation (Pizzorusso et al., 1999). In the present paper, we show that a local supply of NGF inhibits LTP at an early stage of postnatal development. We hypothesize that when NGF concentration increases, synaptic strengthening is inhibited, the degree of plasticity of visual cortical neurons is reduced, and, consequently, sensory deprivation is no longer effective. The sequestering of freely diffusible NGF and/or blockade of TrkA receptors are able to reinduce LTP at an

age when it is normally absent. These results suggest that NGF effects on LTP are relevant to the length of critical period for monocular deprivation. Interestingly, NGF chronic blockade, by intraventricular implants of hybridoma cells producing inhibiting antibodies to NGF, delays the end of the critical period for monocular deprivation (Domenici et al., 1994). Although results obtained in vitro and in vivo point to similar conclusions, one should consider possible discrepancies arising from (1) different mechanisms underlying synaptic plasticity (LTP) and monocular deprivation effects and (2) acute and local versus chronic and systemic NGF/TrkA treatments.

Recently, it has been reported that BDNF exogenous supply affects the development of ocular dominance columns (Cabelli et al., 1995) and enhances LTP in the visual cortex (Akaneya et al., 1997). Although common mechanisms in the ability of different neurotrophins to modulate postnatal development and plasticity of the visual cortex cannot be excluded, it is likely that NGF and BDNF may have two separate types and loci of effects (reviewed by McAllister et al., 1999), whose detailed understanding deserve future investigation.

The Cholinergic System Modulates LTP

To investigate a possible mechanism by which NGF modulates synaptic plasticity in the visual cortex, we assessed the role of the cholinergic system in the modulation of LTP by using the agonist and antagonist of muscarinic receptors, the metabotropic subclass of acetylcholine (ACh) receptors. Muscarinic cholinergic receptors account for five different subtypes (M_{1-5}), coupled to different second messengers and distributed both at pre- and postsynaptic sites (Caulfield and Birdsall, 1998). It is generally accepted that M_1 receptors are located at postsynaptic sites (Levey et al., 1991; Mrzljak et al., 1993). The localization of M_2 receptors is less clearly defined. Traditionally, they have been localized to presynaptic boutons acting as negative feedback autoreceptors. However, several electron microscopy studies targeted M_2 receptors also on postsynaptic pyramidal and nonpyramidal neurons (Rouse et al., 1997; Mrzljak et al., 1998). In addition, Auerbach and Segal (1996) described an LTP form dependent on activation of M_2 postsynaptic receptors in hippocampus. Such difficulties in discriminating pure presynaptic versus postsynaptic effects and the poor selectivity of pharmacological tools acting on different M receptor subtypes prompted us to use agonist/antagonist recognizing all subclasses of muscarinic receptors. We found that blockade of muscarinic receptors by atropine affects LTP at P16–P18. Conversely, activation of muscarinic receptors by muscarine leaves LTP unchanged at P16–P18, while it rescues LTP at P30–P35. The cholinergic system is able to modulate LTP in the visual cortex and in other central nervous structures, as has been previously reported (Sato et al., 1987; Brocher et al., 1992; Sokolov and Kleschevnikov, 1995). Here, we add a new piece of evidence by showing that (1) an antagonist of muscarinic receptors completely inhibits LTP maintenance at P16–P18, mimicking the NGF inhibitory effect, and (2) an agonist of muscarinic receptors rescues LTP at P30–P35, similar to LTP effects induced by blockade of NGF and TrkA receptors.

Interaction between the Cholinergic System and NGF

NGF action on LTP can be mediated through TrkA receptors, the low-affinity p75 receptor, or a combination of both. LTP rescue, at an age when this plastic property is normally lost, is mediated by TrkA receptors, as we have shown by using the specific monoclonal antibody against this receptor. TrkA receptors are present in visual cortex (Pizzorusso et al., 1999), with a fiber-like pattern of distribution. TrkA receptors are also widely expressed in the cholinergic neurons of BF nuclei, including the nucleus Basalis and the diagonal band of Broca (Steininger et al., 1993; Sobreviela et al., 1994; Li et al., 1995; Molnar et al., 1998), which send a well-described cholinergic projection to many cortical areas, including the visual cortex. Thus, it seems likely that TrkA receptors are localized on BF cholinergic fibers innervating visual cortex and that these fibers are the potential target for NGF action.

At P30–P35, we showed that blockade of endogenous NGF, as well as activation of muscarinic receptors, is able to rescue LTP, while blockade of muscarinic receptors cannot do so. We repeated similar experiments, this time using the TrkA-IgG immunoadhesin and the antagonist of muscarinic receptors, atropine, at the same time. Simultaneous local delivery of these compounds leads to an inability to restore LTP in response to HFS at P30–P35. These results suggest that NGF dependence of LTP is mediated by the cholinergic system. However, we cannot completely rule out the alternative possibility that the cholinergic system and NGF signaling control mutually independent mechanisms that act on LTP expression and lead to analogous effects.

Although the exact site(s) and mechanism of interaction between NGF and the cholinergic system remain to be determined, a few obvious possibilities can be considered. In our experimental conditions, NGF could have a modulatory downregulating effect on the cholinergic system. NGF could reduce ACh release simply by binding TrkA receptors on BF cortical fibers, thereby activating an intracellular second messenger cascade interfering with ACh release and/or turnover. This hypothesis is difficult to reconcile with findings showing that NGF increases ACh release in synaptosomes (Knipper et al., 1994; Sala et al., 1998) and in the whole cortex (Maysinger et al., 1992). Besides these results, there is an extensive literature reporting a NGF-mediated increase of choline acetyltransferase (ChAT), in agreement with the idea that NGF induces an upregulation of the cholinergic system. All of these findings suggest that local delivery of NGF might lead to an increase of ACh release in visual cortex slices. At the postsynaptic site, ACh would activate both excitatory and inhibitory neurons. Indeed, muscarinic receptors are expressed in pyramidal cells as well as in different subsets of GABAergic interneurons (Mrzljak et al., 1993; Kawaguchi, 1997; Xiang et al., 1998). LTP inhibition by GABA is well documented in visual cortex (Artola and Singer, 1987; Kirkwood and Bear, 1994). In this context, it will be of great interest to know whether NGF action is mediated by muscarinic activation of GABAergic interneurons and/or pyramidal neurons. Finally, NGF could act

on postsynaptic neurons to regulate directly the intracellular signals underlying synaptic potentiation, possibly interfering with the cholinergic modulated intracellular pathway. A few laboratories have reported that TrkA mRNA is expressed in visual cortex, although at a low level (Valenzuela et al., 1993; Cellerino and Maffei, 1996), and an action of NGF in intrinsic neurons has been documented in organotypic cultures of developing visual cortex (McAllister et al., 1995).

Experimental Procedures

Immunoadhesins and αTrkA Production

αTrkA (Cattaneo et al., 1999) and 9E10 αMyc (Evan et al., 1985) mouse hybridoma cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS) in a 5% CO_2-humidified incubator at 37°C. After reaching a high density, hybridoma cells were shifted to serum-free medium for 2 days, and supernatants were collected for antibody purification by adsorption on protein A–Sepharose columns and eluted with 10 mM HCl. Each fraction was neutralized with 0.5 M Tris-Cl buffer (pH 9).

The DNA sequences coding for the extracellular portion of the human TrkA and TrkB receptors were fused to the Fc portion of camel IgG_2, composed of a 35 amino acid long hinge followed by the C_H2 and C_H3 domains (Hamers-Casterman et al., 1993). The resulting DNA sequences coding for human TrkA and TrkB immunoadhesins were inserted in the Baculovirus (bv) genome (*Autographa californica* nuclear polyhedrosis virus), using a pAcGP67B transfer vector for expression in insect cells. Sf9 insect cells were used to amplify viruses. High five (H5) insect cells were then infected with amplified recombinant bv, and immunoadhesins were purified from cell culture medium by affinity chromatography on a protein A–Sepharose column. Sf9 cells were cultured in TNM-FH medium with 10% FCS, while H5 were cultured in Ex-Cell 400 medium, both in an incubator at 27°C.

ELISA Assay

Plate wells were coated with 1 μg/ml TrkA-IgG or TrkB-IgG (70 μl/well) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Unreacted sites were blocked with phosphate-buffered saline (PBS) + 2% bovine serum albumin (BSA) + 10% FCS for 2 hr at 37°C. Plates were washed with PBS. After 2 hr of incubation with different concentrations of NGF or BDNF (10, 100, 500 ng/ml, Alomone Labs, Jerusalem, Israel), wells were washed three times with PBS + 0.05% Tween 20 and three times with PBS. αD11 (specific antibody against NGF; 1 μg/ml, 70 μl/well) (Cattaneo et al., 1988) or anti-BDNF (preadsorbed to immunoadhesins coated on different plates in order to avoid nonspecific recognition and decrease background) (1:200) diluted in PBS + 2% BSA + 10% FCS were added and incubated for 1 hr at room temperature. After six washes, IgG biotin-conjugated anti-mouse and anti-chicken (1:500) were added and incubated for 1 hr at room temperature; then, plates were washed six times, and peroxidase-conjugated ABC kit (1:1000 in PBS) was added and incubated for 40 min at room temperature. After four washes, substrate solution (70 μl/well) (3,3′, 5,5′-tetramethyl benzidine) was added, and optical density was read at 450 nm wavelength.

To assess the specificity of αTrkA, plate wells were coated as previously described with different concentrations of TrkA-IgG and TrkB-IgG (100 ng/ml, 1 μg/ml), and unreacted sites were blocked. After four washes with PBS, different concentrations (1 μg/ml, 5 μg/ml) of αTrkA in PBS + 2% BSA + 10% FCS were added. After 2 hr of incubation at room temperature, plates were washed with PBS + 0.05% Tween 20 and PBS. Biotin-conjugated anti-mouse IgG preadsorbed to immunoadhesins for 2 hr at room temperature was added (1:500 in PBS). After 1 hr of incubation at room temperature, plates were washed six times. The steps that followed were as previously described.

Den.NGF (80°C for 10 min, 500 ng/ml) was tested for its ability to bind αNGF and TrkA-IgG by two-site ELISA. Methodology of detection was as described above, but horseradish peroxidase–conjugated αNGF (1:2000) was used.

Histochemistry

Brain slices previously treated with biocytin (Sigma, St. Louis, MO) were fixed in 4% paraformaldehyde in PBS for 6 hr at 4°C, then cryoprotected overnight at 4°C in 20% sucrose in PBS (pH 7.4). From brain slices, 50 μm thick sections were cut with a sliding microtome and washed in PBS. Sections were incubated in PBS + 2% H_2O_2 for 20 min at room temperature, then washed three times in PBS (pH 7.4). Nonspecific sites were blocked with a 30 min incubation in PBS + 0.05% Triton X-100 + 5% normal goat serum. After three washes in PBS, sections were incubated in alkaline phosphatase–conjugated ABC kit (1:100 in PBS) for 1 hr at room temperature. After two washes in PBS and two washes in buffered solution (pH 9.5), sections were incubated in developing buffer + nitroblue tetrazolium (75 mg in 1 ml 70% dimethylformamide) + 5-bromo-4-chloro-3-indolylphosphate toluidinium (50 mg in 1 ml 100% dimethylformamide) for no more than 10 min. Reaction was stopped by PBS wash.

Cell Cultures and Bioassays on PC12 Cells

Rat PC12 cells (Greene and Tischler, 1976) were cultured in RPMI 1640 medium with 5% FCS and 10% heat-inactivated horse serum (HS) in a 5% CO_2-humidified incubator at 37°C. For survival and differentiation experiments, PC12 cells were centrifuged, washed with serum-free medium, and plated in collagen-coated 35 mm petri dishes at a density of 4×10^5 cells/dish in RPMI medium with 1% HS. PC12 cells were maintained for 1 week in a 5% CO_2-humidified incubator at 37°C. Negative control cells were cultured in 1% HS, while positive control cells were cultured in 1% HS with 20 ng/ml NGF. TrkA-IgG- and TrkB-IgG-treated cells were incubated for 1 week either in 1% HS with 20 ng/ml NGF and 3 μg/ml immunoadhesins or in immunoadhesins alone. αTrkA and 9E10 αMyc-treated cells were incubated in 1% HS with 20 ng/ml NGF and 4 μg/ml antibodies for 1 week. Moreover, αTrkA-treated cells were incubated in the absence of NGF. Heat-inactivated NGF-treated cells were incubated in 1% HS with 20 ng/ml Den.NGF. In all cases, fresh medium was replaced every 2 days. We considered differentiated all neurons that had processes extending longer than two cell diameters. Results were expressed as PDC with respect to total number of cells in the field. Different fields were randomly chosen (40–50 cells/field).

Slice Preparation

Primary visual cortex slices were prepared from either P16–P18 or P30–P35 Wistar rats. Animals were deeply anesthetized by urethane intraperitoneal injection and then decapitated. The brain was rapidly removed, and coronal sections of the occipital poles (400 μm thick) were sliced by a vibratome. All steps were performed in icy artifical cerebral spinal fluid (ACSF) solution (126 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose) bubbled with 95% O_2/5% CO_2. Slices were stored prior to recording for at least 1 hr in a recovery chamber containing oxygenated ACSF solution, at room temperature. During electrophysiological recordings, slices were perfused at 4 ml/min by oxygenated ACSF, at 33°C ± 1°C.

Electrophysiological Recordings

fEPSPs were evoked by a tungsten concentric bipolar stimulating electrode placed at the WM/layer VI border. The recording electrode was placed in layer II/III. HFS (three trains of 100 pulses at 100 Hz, 10 s interval) was used to induce LTP, whereas LTD was evoked by LFS (900 pulses at 0.1 Hz). Exogenous NGF (Alomone Labs) and Den.NGF were administered at 100 ng/ml. To block the action of endogenous NGF, we used a neutralizing monoclonal antibody against TrkA receptor (αTrkA, 5 μg/ml) and immunoadhesins (TrkA-IgG, 1 μg/ml; TrkB-IgG, 1 μg/ml). To check possible involvement of the cholinergic system, we employed the agonist and antagonist of muscarinic receptors, such as muscarine (100 μM, RBI, St. Louis, MO) and atropine (100 μM, RBI), respectively. NGF, immunoadhesins, αTrkA, muscarine, and atropine were dissolved in 1 M NaCl solution and delivered through the recording pipette, starting 25–35 min before applying HFS or LFS protocols. As a general rule, we used a 10-fold higher concentration of drugs than that commonly employed in bath application experiments.

Changes in the amplitude of fEPSP mirror changes in the slope of the negative potentials and correlate with changes in the magnitude of a monosynaptic current sink (reviewed by Mitzdorf, 1985). Thus, the amplitude of the negative field potential in layer II/III was used as a measure of the evoked population excitatory current. Baseline responses were obtained with a stimulation intensity that yielded 50%–60% of maximal amplitude. All fEPSPs had a peak latency ranging from 5 to 7 ms after stimulation and a maximal amplitude of at least −0.6 mV. fEPSP amplitude was monitored every 15 s and averaged every four responses. Amplitude of fEPSP was measured for each kind of treatment by averaging 3 min of monitored fEPSP centered on 50 min after protocol administration. Values were expressed as mean ± SEM percentage change relative to the mean baseline amplitude. Statistical comparison between control and treated slices was done by applying the nonparametric statistical Rank Sum test. Differences were considered significant when $p \leq 0.05$.

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