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INFUSION OF NERVE GROWTH FACTOR (NGF) INTO KITTEN VISUAL CORTEX INCREASES IMMUNOREACTIVITY FOR NGF, NGF RECEPTORS, AND CHOLINE ACETYLTRANSFERASE IN BASAL FOREBRAIN WITHOUT AFFECTING OCULAR DOMINANCE PLASTICITY OR COLUMN DEVELOPMENT

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Abstract—Intracerebroventricular or intracortical administration of nerve growth factor (NGF) has been shown to block or attenuate visual cortical plasticity in the rat. In cats and ferrets, the effects of exogenous NGF on development and plasticity of visual cortex have been reported to be small or nonexistent. To determine whether locally delivered NGF affects ocular dominance column formation or the plasticity produced by monocular deprivation in cats at the height of the critical period, we infused recombinant human NGF into the primary visual cortex of kittens using an implanted cannula minipump. NGF had no effect on the normal developmental segregation of geniculocortical afferents into ocular dominance columns as determined both physiologically and anatomically. The plasticity of binocular visual cortical responses induced by monocular deprivation was also normal in regions of immunohistochemically detectable NGF infusion, as measured using intrinsic signal optical imaging and single-unit electrophysiology. Immunohistochemical analysis of the basal forebrain regions of the same animals demonstrated that the NGF infused into cortex was biologically active, producing an increase in the number of NGF-, TrkA-, $p75^{NTR}$-, and choline acetyltransferase-positive neurons in basal forebrain nuclei in the hemisphere ipsilateral to the NGF minipump compared to the contralateral basal forebrain neurons.

We conclude that NGF delivered locally to axon terminals of cholinergic basal forebrain neurons resulted in increases in protein expression at the cell body through retrograde signaling. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: TrkA, p75, choline acetyltransferase, retrograde transport, acetylcholine, monocular deprivation.

Monocular deprivation (MD) is a well-studied model of experience-dependent cortical plasticity. When one eye of an animal is occluded during a critical period of development, that eye loses most of its ability to drive neurons in primary visual cortex, shifting the distribution of visual responses to favor the nondeprived eye (Wiesel and Hubel, 1963). Intracerebroventricular (ICV) administration of the neurotrophin nerve growth factor (NGF) to rats during a critical period of development blocks the effects of MD (Domenici et al., 1991; Maffei et al., 1992). This has been demonstrated at several levels of analysis, including physiological, anatomical, immunocytochemical, and behavioral (for a review, see Cellerino and Maffei, 1996). Infusion of NGF directly into developing rat visual cortex also prevents physiological ocular dominance plasticity (Lodovichi et al., 2000).

In addition, anti-NGF antibodies prevent normal anatomical and physiological development of the visual system when they are administered to rats by implantation of antibody-secreting hybridoma cells in the lateral ventricle (Berardi et al., 1994). These antibodies also prolong the critical period for ocular dominance plasticity in the rat (Domenici et al., 1994a). Together, these results demonstrate an important role for endogenous NGF in rat visual cortical development and plasticity.

In contrast to the rat, the effects in the kitten of exogenous NGF on physiological plasticity following MD have been relatively small (Carmignoto et al., 1993) or nonexistent (Galuske et al., 2000). Also, delivery of NGF using osmotic minipumps did not disrupt the final stages of the segregation of thalamocortical afferents into ocular dominance columns in the cat (Cabelli et al., 1995), while focal injection of microspheres coated with NGF into visual cortex had no effect on the atrophy of lateral geniculate nucleus (LGN) neurons representing the

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Abbreviations: BI, bias index; BSA, bovine serum albumin; CBI, contralateral bias index; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine; ICV, intracerebroventricular; LGN, lateral geniculate nucleus; MD, monocular deprivation; MI, monocularity index; NGF, nerve growth factor; P, postnatal; PB, phosphate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TBS, tris-buffered saline.

deprived eye in the ferret, in contrast to treatment with neurotrophin-4/5 (Riddle et al., 1995). Although exogenous NGF seems to be fairly ineffective in the developing cat and ferret, infusion of NGF into primary visual cortex of adult cats in combination with MD caused a paradoxical shift in visual responses towards the deprived eye (Gu et al., 1994; Galuske et al., 2000), even though MD of normal adult cats does not result in ocular dominance plasticity (Hubel and Wiesel, 1970).

We infused NGF locally into visual cortex of kittens during the period of ocular dominance column formation. Columns were assessed using single-unit electrophysiology and transneuronal labeling of intraocularly injected radiolabeled proline. In addition, we determined the effect of local cortical delivery of NGF on ocular dominance plasticity with single-unit electrophysiology and intrinsic signal optical imaging. Effects of the infused NGF on protein expression were tested by measuring immunoreactivity for NGF, the low-affinity NGF receptor $p75^{NTR}$ (Chao et al., 1986), the high-affinity NGF receptor TrkA (Kaplan et al., 1991; Klein et al., 1991), and the acetylcholine synthetic enzyme choline acetyltransferase (ChAT) in basal forebrain cholinergic neurons that project to the cortex of the infused hemisphere.

Some of these data have been published previously in abstract form (Silver et al., 2000).

EXPERIMENTAL PROCEDURES

Animals

Sixteen kittens were used for this study. All of them were obtained from the breeding colony at the University of California (San Francisco, CA, USA) and had normal pigmentation. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1996) and were approved by the Committee on Animal Research, University of California. Every effort was made to minimize the number of animals used in these experiments. In addition, the suffering of the experimental animals was reduced through the use of anesthetics and analgesics.

Implantation of cannula minipumps

Osmotic minipumps containing NGF were used for two types of experiments. In MD experiments, model 1007D 1-week minipumps (flow rate 0.5 μl/h; Alza, Palo Alto, CA, USA) or model 2002 2-week pumps (flow rate 0.5 μl/h) were used. For ocular dominance column development experiments, model 2002 2-week minipumps were coated in paraffin to decrease the flow rate and extend the duration of NGF administration to about 4 weeks. In both cases, the pumps were filled with 0.2 mg/ml recombinant human NGF (supplied by Genentech). The stock solution of NGF (1 mg/ml) contained either 10 mM sodium acetate or 10 mM sodium succinate as a buffer (pH = 5.0), and 100 mM sodium phosphate buffer (PB) and sodium chloride were added so that the final buffer concentration was 10 mM and the final sodium concentration was 150 mM. The pump also contained 0.1% (1 mg/ml) bovine serum albumin (BSA) to decrease nonspecific binding of the NGF to the pump and tubing. Vehicle solutions contained the same concentration of buffers, salts, and BSA and had the same pH as the NGF solutions. Animals were anesthetized with a mixture of halothane and N_2O/O_2 (1:1). Minipumps were attached to 30-gauge cannulae and, using sterile surgical technique, were implanted 2 mm beneath the cortical surface in the lateral gyrus (Horsley-Clarke coordinates mediolateral 2.0, anterioposterior 0.0). Following recovery of the animal, amoxicillin was administered orally. In some of the experiments in which a cannula minipump containing vehicle solution was implanted in the contralateral hemisphere, the experimenters were blind to the contents of the minipumps until the entire experiment had been completed.

Transneuronal transport and autoradiography

Kittens at ages postnatal (P)29–P34 were anesthetized with a mixture of halothane and a 1:1 ratio of N_2O/O_2, and an incision was made in the lateral canthus of the eye. The exposed eyeball was punctured with a sterile 30-gauge needle. This allowed the insertion of a 50-μl Hamilton syringe containing [2,3,4,5-^{3}H]proline (specific activity 98 Ci/mmol; Amersham, Arlington Heights, IL, USA). 2 mCi of proline in 20 μl of either distilled water or physiological saline was slowly injected into the vitreous humor 4 to 5 mm from the scleral surface. The needle was left in place for 30 s following delivery of the proline, and a Q-tip was applied to the scleral puncture immediately following the withdrawal of the needle to prevent and capture any leakage from the vitreal chamber. Mattress sutures were used to close the lateral canthus incision.

Following a survival time of 10–12 days to allow transneuronal transport of the tritiated label to geniculocortical terminals, animals were prepared for single-unit electrophysiology (described below). Following the end of the recording session, animals were perfused with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) for 3 min followed by 0.1 M PBS. Primary visual cortex was removed and flattened between two glass plates by making relieving cuts in the occipital pole of the cortex. While the tissue was flattening it was postfixed in 4% paraformaldehyde/30% sucrose in 0.1 M PB (pH = 7.4). Tangential sections 40 μm thick were cut using a freezing microtome. Sections were washed at least three times with distilled water, mounted onto gelatinized glass microscope slides from 0.9% NaCl, cleared in xylenes, and processed for autoradiography. Sections were photographed through a macroscope using dark-field optics, and the images collected from adjacent sections were montaged to visualize the overall pattern of labeled geniculocortical afferents in layer IV.

MD

Kittens ranging in age from P28 to P31 were anesthetized with a mixture of halothane and N_2O/O_2 (1:1). The eyelids were trimmed along the margins with surgical scissors and sutured together with three or four mattress sutures of #4 vicryl. A window was left on the medial side to allow lachrymal secretions. The sutured eye was treated with an antibiotic ointment containing bacitracin, neomycin, and polymyxin, and oral amoxicillin was administered postoperatively to prevent infection.

Single-unit electrophysiology

Animals were given a subcutaneous injection of atropine (0.1 mg/kg) to inhibit respiratory secretions. Anesthesia was induced using a combination of halothane and N_2O/O_2 (1:1). The femoral vein was cannulated with 50-gauge polyethylene tubing. At this point, halothane was discontinued and anesthesia was maintained by intravenous administration of pentobarbital. A tracheotomy was performed, the trachea was cannulated, and a mixture of N_2O and O_2 (2:1) was delivered through the trachea tube for the duration of the experiment. Body temperature was maintained at 38°C using a feedback controlled heating pad connected to a rectal thermometer.

Animals were placed in a stereotaxic head holder, and opthalmic atropine sulfate (1%) was applied to the eyes to maximally dilate the pupils. Opthalmic phenylephrine hydrochloride (10%) was also administered to the eyes to retract the nictitating membranes. A bilateral craniotomy exposing both lateral gyri was

performed over primary visual cortex. Animals were then paralyzed by intravenous delivery of gallamine triethiodide (10 mg/kg/h) dissolved in 5% Ringer's solution with dextrose and were switched to artificial respiration. Following an initial bolus of 15 mg/kg, the dura was retracted, and gallamine was continuously administered at a rate of 1–2 ml/kg/h. The respiratory rate and volume were adjusted to result in an end-tidal expired CO_2 concentration of approximately 4% as measured with a capnograph. Contact lenses were used to protect the corneas and to focus the eyes on a tangent screen where the visual stimuli were delivered. The locations of the optic discs were plotted on the screen using an opthalmoscope.

Anesthesia was continued for the duration of the experiment by delivering Nembutal intravenously as needed. Level of anesthesia was determined by (1) monitoring end-tidal expired CO_2 levels, (2) visual inspection of the spectral content of electroencephalography (EEG) recordings made from a supradural electrode implanted in a small hole drilled through the skull, (3) heart rate measurements, and (4) observing whether there was an increase in heart rate following a paw pinch or turning on the room lights.

Tungsten microelectrodes were advanced down the medial bank of the lateral gyrus in increments of 100 μm using a stepping motor microdrive. Visual stimuli were delivered using a hand-held slit lamp. For each unit, receptive field location, ocular dominance, orientation preference and selectivity, visual responsiveness, and habituation in response to optimal stimulation with bars of light were recorded. Each unit was assigned to one of seven ocular dominance categories (Hubel and Wiesel, 1962) in which 1 indicates visual responsiveness to only the deprived eye, 7 represents visual responsiveness to only the nondeprived eye, 4 is equal strength of response to monocular stimulation of the two eyes, and 2, 3, 5, and 6 are intermediate categories. The amount of shift in ocular dominance induced by MD for a population of neurons is referred to as the bias index (BI) and was quantified using the following formula:

$$\mathrm{BI} = [(\mathbf{7}-\mathbf{1}) + ((2/3)(\mathbf{6}-\mathbf{2})) + ((1/3)(\mathbf{5}-\mathbf{3})) + n]/2n$$

Here, bold numbers indicate the number of units in each ocular dominance category, and n represents the total number of units in the population for which an ocular dominance value could be assigned. The BI would have a value of 0 if the cortex contained units that only responded to the deprived eye, and it would be 1 if the ocular dominance shift were complete (if all units responded solely to visual stimulation of the nondeprived eye).

To quantify the developmental transition from binocularity to monocularity in kitten visual cortex, a monocularity index (MI) was employed (Stryker and Harris, 1986). Again, seven ocular dominance categories were used, but in this case, category number 1 contained units which responded only to visual stimulation of the eye contralateral to the hemisphere being recorded, category 7 had units responsive only to ipsilateral stimulation, and category 4 contained cells that were completely binocular. The MI is defined as:

$$\mathrm{MI} = [(\mathbf{1}+\mathbf{7}) + ((2/3)(\mathbf{2}+\mathbf{6})) + ((1/3)(\mathbf{3}+\mathbf{5}))]/n$$

The terms in this formula are the same as those used for the BI above. A MI of 0 would indicate that all units were in ocular dominance category 4 (driven equally well by monocular visual stimulation of either eye), while a MI of 1 would be obtained if all units were strictly monocular (in ocular dominance category 1 or 7).

Intrinsic signal optical imaging

A description of the basic principles underlying intrinsic signal optical imaging of responses in visual cortex of anesthetized paralyzed animals can be found in Bonhoeffer and Grinvald (1996). In brief, presentation of large oriented visual stimuli causes a decrease in the reflectance of responsive regions of primary visual cortical tissue that correlates with synaptic activation of visual cortical neurons (Grinvald et al., 1986). Acquisition of images of the exposed visual cortex during repeated monocular presentation of oriented visual stimuli allows the derivation of a map of oriented responses in visual cortex.

The image collection and processing techniques used in this study have been described in detail by Issa et al. (1999) and will only be summarized here. Animals were prepared as described above for single-unit electrophysiology. Following retraction of the dura, the cortex was covered with a large mound of 3% agarose in physiological saline and sealed with a glass cover slip surrounded by small strips of cellophane to prevent drying of the agarose. Full field high contrast square wave gratings at either four or eight evenly spaced orientations were monocularly presented on a video monitor in pseudorandom order. Images of reflectance at 610 nm were collected using a CCD camera (Princeton Instruments). The focal plane was 400 μm below the vasculature on the cortical surface. Images were high-pass-filtered (1.2 mm kernel) to correct for DC changes in response size during the course of the experiment. Responses were normalized by dividing by the response to presentation of a gray screen (blank screen normalization). Angle maps were derived by computing the vector sum of the responses to the set of eight oriented gratings for each pixel and assigning a color corresponding to the angle of the vector sum (Blasdel and Salama, 1986).

The effects of MD were assessed by computing an optical contralateral BI for each imaged hemisphere (optical contralateral bias index (CBI), Issa et al., 1999). This index would have a value of 0 if the cortex were completely dominated by the eye ipsilateral to the imaged hemisphere and a value of 1 if the contralateral eye were totally dominant. Because normal animals have a slight contralateral bias, their optical CBI has a value of approximately 0.55 (Crair et al., 1998). To compute deviations from this normal value following MD, a plasticity index was defined that corresponds to the absolute value of the difference between the recorded optical CBI in a given hemisphere and the average optical CBI in normal animals (0.55). This results in a measure of the strength of ocular dominance plasticity that allows the direct comparison of NGF-infused and vehicle-infused hemispheres.

Immunohistochemistry and cell counts

Animals were transcardially perfused with 1 l 0.1 M PB (pH 7.4) followed by 1 l 4% paraformaldehyde in the same buffer. All solutions were used at 4°C. The brains were blocked using stereotaxic coordinates (Horsley-Clarke anterioposterior +9.0) to separate basal forebrain nuclei from the visual cortex. Blocks were removed from the animal and postfixed in 4% paraformaldehyde in PB for 1 to 3 h and then embedded in 5% agar in distilled water. 70–80 μm sections were cut on a Vibratome and stored in PB+0.9% NaCl (PBS)+0.05% thimerosal. In some cases, endogenous peroxidase activity was quenched by incubating in 1% hydrogen peroxide in PBS for 15 min followed by five washes of 10 min each in PBS. Sections were incubated in a blocking solution consisting of PBS with 20% donkey serum, 2% BSA, 5% sucrose, 0.5% Triton X-100, and 0.05% thimerosal. For some experiments, endogenous avidin binding sites were blocked using an avidin/biotin blocking kit (Vector, Burlingame, CA, USA). Sections were incubated in primary antibodies (rat anti-NGF, 1:2000, Promega, Madison, WI, USA; affinity-purified rabbit anti-TrkA, 1:1000, RTA; affinity-purified rabbit anti-p75NTR, 1:1000, REX; or goat anti-ChAT, 1:1000, Chemicon, Temecula, CA, USA) for 48 h at 4°C in blocking solution. Following three washes of 10 min each in PBS, sections were transferred to either horseradish peroxidase-conjugated (Jackson, West Grove, PA, USA) or biotinylated secondary (Vector) antibodies and were incubated overnight at 4°C in blocking solution. The sections with biotinylated secondary antibodies were washed for 10 min each in PBS and then labeled with peroxidase using the Vector ABC kit. All sections were then washed three times for 10 min each in 10 mM Tris buffer+0.9% NaCl (TBS). Immunoreactivity was visualized by incubating sections in 0.5 mg/ml 3,3′-diaminobenzidine (DAB), 6.95 mg/ml nickel ammonium sulfate, and 0.03% hydrogen peroxide

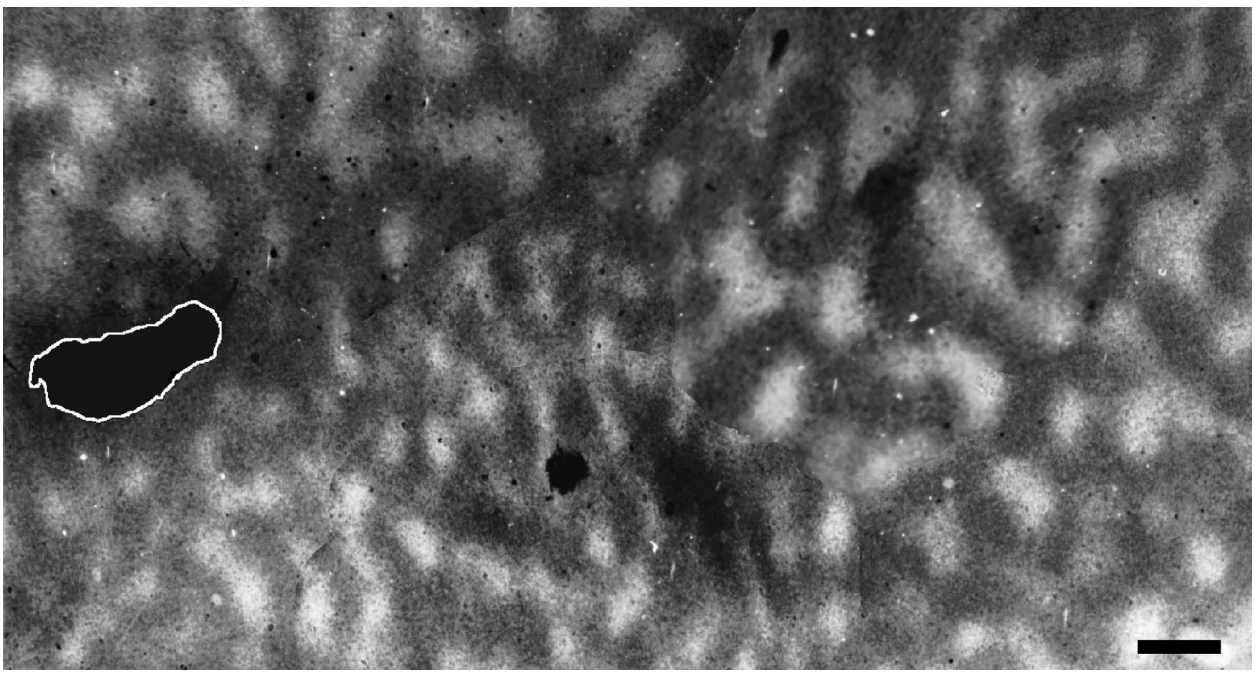

Fig. 1. Local cortical delivery of NGF does not block ocular dominance column formation. NGF was infused continuously into primary visual cortex during the period of ocular dominance column development. Ocular dominance columns were made visible by injecting tritiated proline into one eye and allowing transneuronal transport of the radioactive label to occur. Tangential sections of flattened visual cortex were exposed for autoradiography and photographed with dark-field optics, so that the labeled geniculocortical afferent terminals appear light. Images from adjacent sections were montaged to create this figure. The lesion in the tissue created by the cannula is outlined in white. Ocular dominance columns can be seen throughout the field and as near as 500 μm from the infusion site. Scale bar = 1 mm.

(30%) in 10 mM TBS. Sections were mounted onto gelatinized glass microscope slides from tap water, dehydrated in a series of graded ethyl alcohols, cleared in xylenes, and coverslipped with DPX.

The boundaries of the septal nuclei, diagonal band of Broca, and substantia innominata were determined based on the atlas and classification system of Berman and Jones (1982). In each basal forebrain tissue section, the numbers of TrkA-, $p75^{NTR}$-, and ChAT-positive neurons in these brain areas were counted for both NGF- and vehicle-infused hemispheres by an observer blind to the side of NGF administration. The values in the two hemispheres were compared for each section using a contrast index: $(N_{NGF}-N_{VEH})/(N_{NGF}+N_{VEH})$, where N_{NGF} is the number of immunopositive cells for a given antigen in a particular brain region in the NGF-infused hemisphere, and N_{VEH} is the corresponding measure in the vehicle-infused hemisphere. This index would have a value of 0 if the numbers of positive cells were equal in the two hemispheres, −1 if all the positive neurons were in the vehicle-infused hemisphere, and 1 if all the positive neurons were in the NGF-infused hemisphere. Each section generated one, two, or three values of the contrast index, depending on how many of the basal forebrain regions (septal nuclei, diagonal band of Broca, substantia innominata) were present in a given section. Average contrast indices were computed for each combination of animal, antigen, and brain region. To test significance of NGF effects, a weighted average of the contrast indices for the different brain regions was derived, and the weights corresponded to the number of cells counted in each brain region. A one-tailed *t*-test was performed to determine whether the weighted averages were significantly different from zero with a probability of less than 0.05. The resulting *P*-values were not corrected for multiple comparisons, since the standard method for accomplishing this (the Bonferroni correction) assumes independence of the different statistical comparisons. However, the presence of strong positive correlations among different brain regions and among different antibodies in our data set precluded the use of the Bonferroni correction.

RESULTS

Local cortical infusion of NGF has no effect on ocular dominance column formation

Previous work by Cabelli et al. (1995) showed that local cortical infusion of NGF did not desegregate ocular dominance columns in the cat. In that study, NGF was delivered from P28 to P42. Since ocular dominance columns are detectable in the cat by the second postnatal week, both anatomically (Crair et al., 2001) and functionally (Crair et al., 1998), the Cabelli et al. study examined whether NGF had any effect on the later stages of ocular dominance column formation. To determine if NGF has a role in the initial segregation of geniculocortical afferents into ocular dominance columns, we infused NGF for 4 weeks at a concentration of 0.2 mg/ml locally into primary visual cortex of one hemisphere beginning at P14–P18. Geniculocortical afferents were visualized with intraocular injections of tritiated proline, a tracer which is transneuronally transported from the eye to axon terminals in layer IV of the cortex serving the injected eye. NGF had no effect on development of ocular dominance columns, as column formation proceeded normally in areas of the primary visual cortex as close as

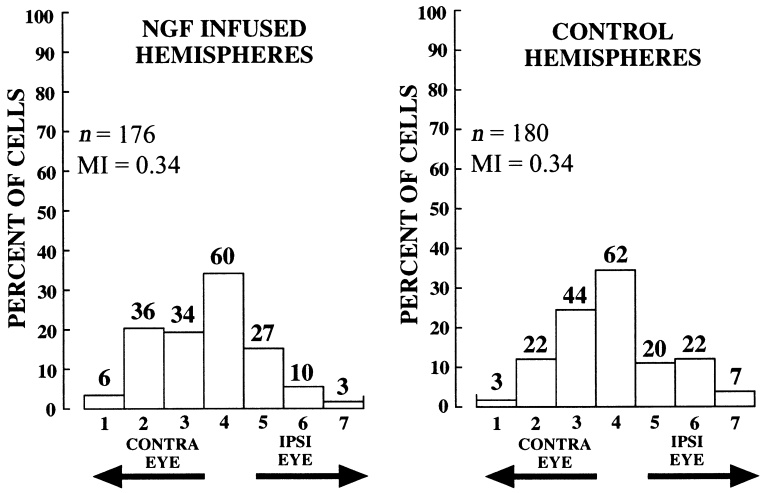

Fig. 2. Local cortical delivery of NGF does not block the normal developmental transition from binocular to monocular visual responses in visual cortical neurons. Extracellular single-unit electrophysiological recordings were made in primary visual cortex of kittens that received local infusion of NGF during the developmental period in which visual responses become more monocular. Ocular dominance histograms show the numbers of cells in each ocular dominance group (Hubel and Wiesel, 1962). A MI was used to quantify the ocular dominance distribution (see Experimental procedures). This index was similar in NGF-treated and control hemispheres.

500 μm to the cannula (Fig. 1). Similar results were obtained in a sample of four animals.

The developmental segregation of geniculocortical afferents into ocular dominance columns is accompanied by a transition from binocular to monocular responses in kitten primary visual cortex (LeVay et al., 1978). This process was also unaffected by infusion of 0.2 mg/ml NGF for 4 weeks into primary visual cortex beginning at P14–P18. Single-unit microelectrode recordings from primary visual cortical neurons were used to assign each cell to one of seven ocular dominance categories (Hubel and Wiesel, 1962) based on their responses to optimally oriented bars of light presented using a hand-held slit lamp. Fig. 2 shows that the MI (see Experimental procedures) of cortical hemispheres infused with NGF was 0.34 (176 neurons from four animals), and the MI of control hemispheres with no implanted minipump was also 0.34 (180 neurons from six animals). Neurons in the NGF-infused hemisphere had qualitatively normal visual responsiveness, habituation to repeated visual stimulation, and selectivity for stimulus orientation and direction of movement (data not shown).

Brief local cortical infusion of NGF has no effect on ocular dominance plasticity

Although injections of NGF into the lateral ventricle blocked ocular dominance plasticity in the rat (Maffei et

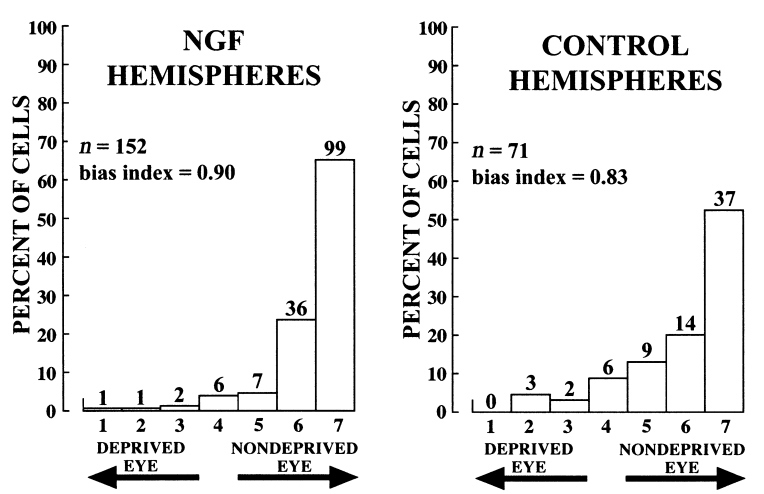

Fig. 3. Brief local cortical delivery of NGF does not block ocular dominance plasticity as assayed by single-unit electrophysiology. NGF was administered to kitten primary visual cortex for a total of 4–5 days during the height of the critical period for ocular dominance plasticity. After 2–3 days of infusion, monocular lid suture was performed, and the deprivation was continued for the latter 2 days of the period of NGF infusion. Ocular dominance plasticity was quantified from histograms by computing a BI (see Experimental procedures). Normal shifts in binocular visual responses towards the nondeprived eye occurred in hemispheres that received NGF and in control hemispheres (receiving either a vehicle solution or no minipump at all).

al., 1992), a similar protocol in the kitten merely attenuated the physiological shift in ocular dominance induced by MD (Carmignoto et al., 1993). In addition, local delivery of NGF had no effect on ocular dominance column formation in the kitten (Figs. 1 and 2; see also Cabelli et al., 1995) or on the MD-induced shrinkage of cell size of LGN neurons representing the deprived eye in the ferret (Riddle et al., 1995). To determine whether local cortical delivery of NGF had any effect on ocular dominance plasticity in the kitten, NGF (0.2 mg/ml) was infused into primary visual cortex of one hemisphere of kittens for 4 or 5 days beginning on postnatal day 25 to 27. Two or three days after the beginning of infusion, monocular lid suture was performed. After 48 h of MD, the effects of the deprivation were assessed with single-unit electrophysiology and intrinsic signal optical imaging. Two days of MD at the height of the critical period causes a saturating or nearly saturating ocular dominance shift in kittens as measured using single-unit electrophysiology (Olson and Freeman, 1975) and intrinsic signal optical imaging (Crair et al., 1997).

The effects of MD were quantified using single-unit electrophysiology. Ocular dominance histograms were generated by recording single-unit visual responses in primary visual cortex and assigning the units to one of seven categories (Hubel and Wiesel, 1962) based on their relative responses to deprived and nondeprived eye stimulation. The sample size consisted of three NGF-infused hemispheres and two control hemispheres. One of these control hemispheres was implanted with a minipump that delivered vehicle solution, and one had no implant. These hemispheres were pooled to generate the control ocular dominance histogram. Some of the units in these histograms have also appeared in a previous publication (Gillespie et al., 2000).

In both NGF-treated and control hemispheres, the ocular dominance shift was normal and complete (Fig. 3). Bias indices, a quantitative measure of ocular dominance plasticity, were generated based on the percentage of neurons in each ocular dominance category (see Experimental procedures), and they were similar for both NGF-infused and control hemispheres: BI = 0.90 for NGF hemispheres and 0.83 for control hemispheres. These results are consistent with previous studies showing no effect of cortical NGF infusion on ocular dominance plasticity in the kitten (Galuske et al., 2000; Gillespie et al., 2000).

NGF also had no effect on the responsiveness of primary visual cortical neurons to visual stimulation or habituation to repeated stimulus presentations. Each unit was rated on a 5-point responsiveness scale (0 is completely unresponsive and 5 indicates strong responses to optimal stimulation) and a 3-point habituation scale (0 is no habituation, and 3 means rapidly and strongly habituating). For the three animals included in Fig. 3, responsiveness was 4.62 ± 0.05 (mean ± S.E.M.) in NGF-infused hemispheres and 4.62 ± 0.07 for control hemispheres. Similarly, NGF-treated hemispheres had a habituation index of 0.99 ± 0.05, and this value was 0.92 ± 0.10 in control hemispheres. These differences between NGF-infused and control hemispheres did not reach statistical significance for either responsiveness or habituation as assessed using a two-tailed *t*-test and a criterion probability of 0.05. Orientation and direction selectivity were not systematically quantified, but there were no obvious qualitative effects of NGF infusion on these response properties.

The amount of plasticity induced by MD was also determined using intrinsic signal optical imaging. This technique measures decreases in cortical reflectance following visual stimulation (Bonhoeffer and Grinvald, 1996). These responses are known to correlate with synaptic activation of cortical neurons (Grinvald et al., 1986). Visual stimulation of the nondeprived eye with oriented full field gratings resulted in specific patterns of activation, and each pixel in the map was assigned a color corresponding to its preferred orientation. This results in an angle map of oriented responses (Blasdel and Salama, 1986) that corresponds to the well-known arrangement of orientation columns in primary visual cortex (Hubel and Wiesel, 1962). Angle maps of orientation selectivity in the visual cortex were generated in the hemisphere that received NGF and compared to contralateral control hemispheres that received a vehicle solution. One correlate of ocular dominance plasticity is the loss of spatial structure in angle maps of orientation selectivity derived by visual stimulation of the deprived eye (Kim and Bonhoeffer, 1994). In NGF-treated hemispheres, the angle maps derived with visual stimulation of the deprived eye show little spatial structure, while maps of the same fields of cortical tissue derived with nondeprived eye stimulation show the normal arrangement of orientation selective responses (Fig. 4).

To quantify the extent of ocular dominance plasticity, plasticity indices were computed for the NGF- and vehicle-infused hemispheres (see Experimental procedures). This index is derived from optical CBIs which are based on pixel by pixel comparisons of the size of response generated by visual stimulation at the optimal stimulus orientation of the nondeprived and deprived eye and correlate well with CBIs measured using single-unit recordings (Issa et al., 1999). A plasticity index of 0 indicates no effect of MD, and larger values correlate with greater amounts of ocular dominance plasticity. For animal K11, the plasticity index was 0.364 in the NGF-infused hemisphere and 0.245 in the vehicle-infused hemisphere. Similarly, for animal K108, the optical CBI was 0.270 in the NGF-infused hemisphere and 0.198 in the vehicle-infused hemisphere. Thus, in both cases, there is substantial ocular dominance plasticity that is not reduced by local cortical delivery of NGF. These results are in agreement with those of Galuske et al. (2000), in which MD of 7–8 days duration was unaffected by simultaneous cortical infusion of NGF in critical period kittens as assessed using intrinsic signal optical imaging.

Prolonged NGF infusion has no effect on ocular dominance plasticity

In the experiments presented in Figs. 3 and 4, the NGF was infused only for 2 or 3 days before the beginning of deprivation, and previous studies of the kitten

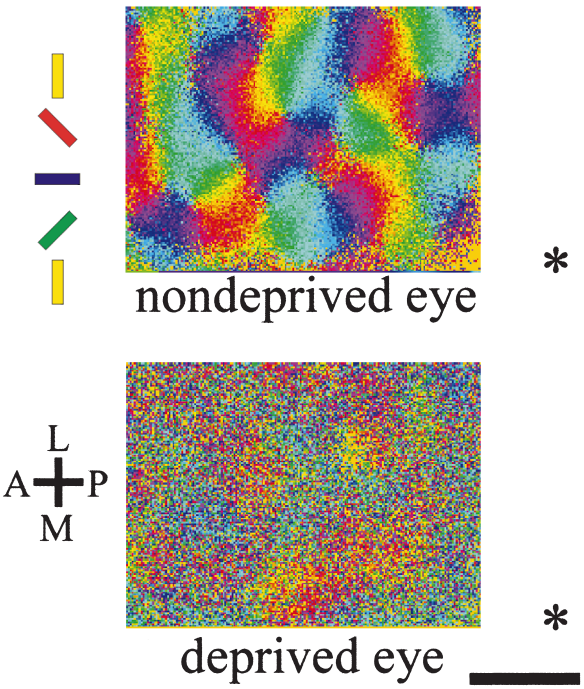

Fig. 4. Brief local cortical delivery of NGF does not block ocular dominance plasticity as assayed by intrinsic signal optical imaging. Animals were administered NGF as in Fig. 3 and monocularly deprived for two days near the peak of the critical period. The angle map derived by visual stimulation of the deprived eye has little spatial structure, while the angle map derived by nondeprived eye visual stimulation shows the characteristic pattern of pinwheels and isoorientation domains (see Experimental procedures). Asterisk indicates location of the infusion cannula, and colored bars at left represent preferred stimulus orientation for each pixel of the cortical maps. The cross shows the orientation of the imaged field, where A is anterior, P is posterior, L is lateral, and M is medial. Scale bar = 1 mm.

started NGF infusion and MD at the same time (Carmignoto et al., 1993; Galuske et al., 2000). However, other experiments have suggested that delivery of exogenous NGF can produce effects that require a period of days or even weeks to be expressed. Three days of intraventricular infusion of NGF in NGF heterozygote knockout mice had no effect on learning in the Morris water maze, but prolonged (5 weeks) administration improved performance (Chen et al., 1997). Similarly, performance of aged rats on a spatial memory task was improved by 4 weeks of ICV delivery of NGF, while 2 weeks was insufficient (Markowska et al., 1996). Finally, Fagiolini and Stryker (1996) found that at least 2 days of ICV injections of NGF before the beginning of MD were necessary to prevent ocular dominance plasticity in the developing mouse.

To test whether prolonged cortical delivery of NGF prior to the start of MD reduced ocular dominance plasticity, NGF was infused into visual cortex of one animal (K34) beginning on P22. This animal was deprived on P27, and single-unit recordings were made starting on P29. Five days of infusion prior to the beginning of MD also had no effect on the size of the ocular dominance shift, as the BI measured by microelectrode recording was 0.92 (Fig. 5A). The effects of prolonged infusion of NGF before MD were also examined with intrinsic signal optical imaging. Animal K34, described above, had a plasticity index in the NGF-infused hemisphere of 0.248. The angle map for this animal derived with deprived eye stimulation had deteriorated, while the nondeprived eye angle map had a normal appearance (Fig. 5B). In addition, we implanted a minipump in one animal (K341) on P27, infused NGF for 10 days, and then monocularly deprived the animal for 2 days with continuing NGF infusion. The plasticity index for the NGF-infused hemisphere was 0.200. Finally, a third animal (K337) received NGF from P22–P38 and was monocularly deprived for the last 2 days of the infusion, resulting in a plasticity index of 0.200. The smaller plasticity indices in K341 and K337 are consistent with their period of MD taking place after the peak of the critical period. The results of these experiments indicate that even long-term cortical infusion of NGF does not prevent ocular dominance plasticity in the kitten.

Immunohistochemical verification of NGF infusion into visual cortex

Since cortical administration of NGF had no effect on ocular dominance column formation or ocular dominance plasticity, it was important to demonstrate that NGF had been successfully delivered to the visual cortices of the animals in which the effects of exogenous NGF had been assayed. The diffusion of NGF into the visual cortex surrounding the cannulae was measured using an anti-NGF antibody and standard DAB immunohistochemistry. The spread of NGF ranged from 1.5 to 4 mm from the cannula tip within the lateral gyrus, and no NGF signal was ever detected in the contralateral hemisphere (Fig. 6A, B).

A small amount of DAB precipitate was observed in the tissue immediately surrounding the cannula in the vehicle-infused hemispheres. This may be due to erythrocytes that entered the tissue as a result of the small amount of local bleeding in brain parenchyma associated with cannula implantation. Erythrocytes have high levels of peroxidase enzyme activity and therefore produce DAB precipitate that is typically a result of antibody-conjugated horseradish peroxidase in these experiments.

A higher power view of the infusion site indicates that exogenous NGF was not taken up by local cortical neurons in large amounts. In fact, the NGF-like immunoreactivity in the area surrounding the infusion cannula seemed to be confined mainly to the cortical neuropil and to avoid cortical neuronal cell bodies (Fig. 6C). The biological activity of the recombinant human NGF used in this study was also tested. The potency of the NGF for promoting neurite outgrowth in pheochromocytoma (PC12) cells was equal to that of NGF that was biochemically purified from mouse salivary gland (data not shown).

Local cortical NGF infusion increases the number of basal forebrain neurons immunoreactive for NGF, TrkA, $p75^{NTR}$, and ChAT

Previous studies demonstrated that basal forebrain

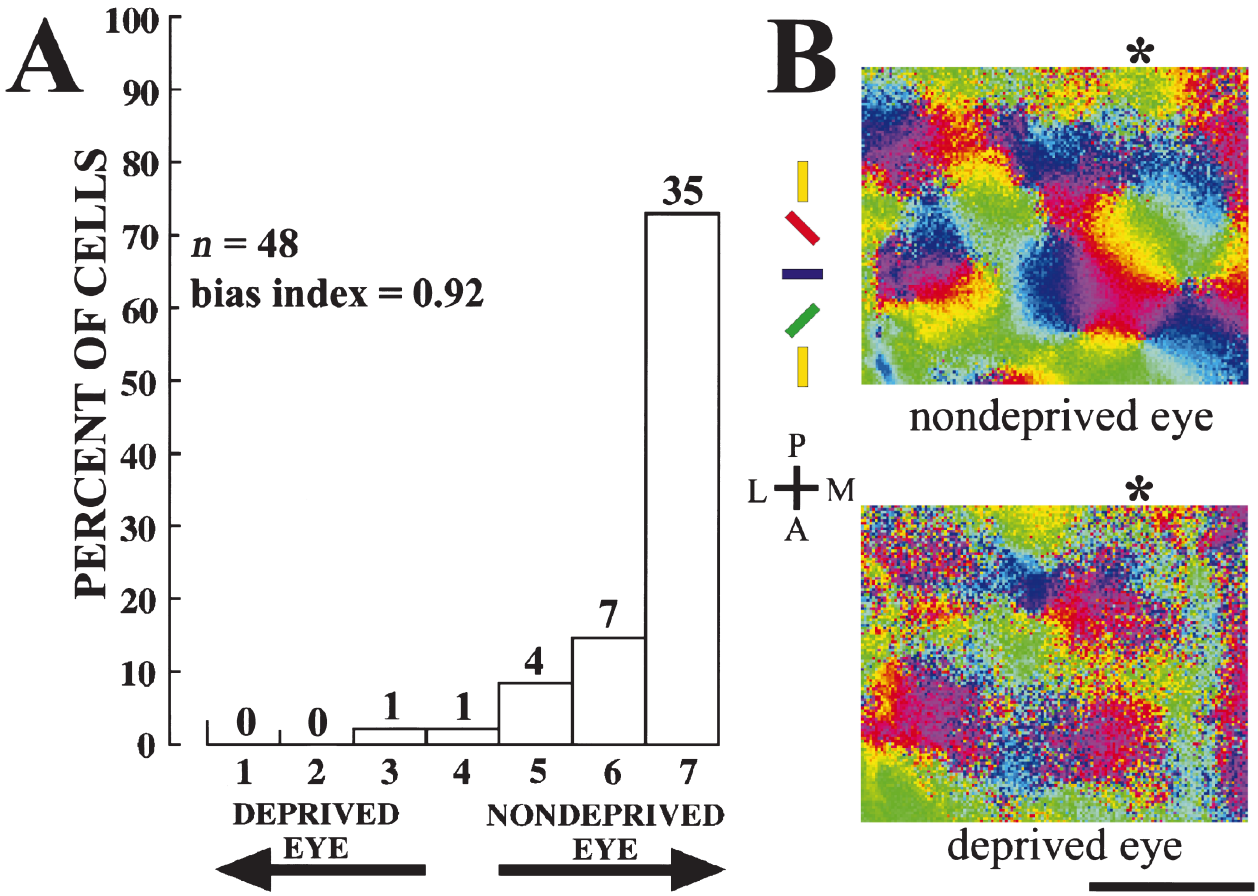

Fig. 5. Prolonged infusion of NGF beginning 1 week before and continuing through the period of MD does not prevent ocular dominance plasticity. (A) This animal received cortical infusion of NGF from P22 to P29 and was monocularly deprived from P27 to P29. The shift in single-unit responses towards the nondeprived eye occurred normally. (B) Intrinsic signal optical images from the visual cortex adjacent to the infusion cannula at asterisk in the same animal. Visual responses were strong and well-organized through the nondeprived eye and weaker and poorly organized through the deprived eye, revealing a powerful effect of deprivation. Colored bars at left indicate preferred stimulus orientation for each pixel of the cortical maps. The cross shows the orientation of the imaged field, where A is anterior, P is posterior, L is lateral, and M is medial. Scale bar = 1 mm.

cholinergic neurons are in a position to mediate effects of NGF infused into the cortex. Neurons in the septal nuclei, diagonal band of Broca, and substantia innominata regions of the basal forebrain project over the entire extent of the cerebral cortex in adult cats (Irle and Markowitsch, 1984). Many basal forebrain neurons express the low-affinity receptor $p75^{NTR}$ (Hefti et al., 1986; Springer et al., 1987) as well as the high-affinity NGF receptor TrkA (Steininger et al., 1993). Almost all neurons expressing NGF receptors are also positive for the cholinergic marker ChAT ($p75^{NTR}$: Dawbarn et al., 1988; Batchelor et al., 1989; TrkA: Sobreviela et al., 1994). In the cerebral cortex, axons that express $p75^{NTR}$ (Pioro and Cuello, 1990) and TrkA (Sobreviela et al., 1994; Hu et al., 1997) probably arise from the basal forebrain, and basal forebrain neurons retrogradely transport NGF exogenously applied to cortex (Seiler and Schwab, 1984; Domenici et al., 1994b).

Since the cortical projections of cat basal forebrain neurons do not cross the midline (Ribak and Kramer, 1982; Irle and Markowitsch, 1984), it is possible to assay the effects of delivering NGF locally to the axons of these neurons by comparing basal forebrain nuclei in a hemisphere in which NGF was infused to the corresponding nuclei in a control hemisphere. In three cats, minipumps containing NGF or vehicle solution were implanted in the two hemispheres as described for the short-term MD experiments above; two of the cats were also used for the physiological recording experiments. Fig. 7A shows the NGF labeling of cells in the septal nuclei in the two hemispheres for a case in which baseline endogenous label in the control hemisphere was below detectable levels. The label ipsilateral to the NGF infusion presumably represents NGF transported retrogradely to these cells from their cortical axon arbors (Seiler and Schwab, 1984; Domenici et al., 1994b). Fig. 7B indicates that more neurons in the septal nuclei are labeled for ChAT on the side ipsilateral to the NGF infusion than on the control side. The two NGF receptors, TrkA and $p75^{NTR}$, also showed more label ipsilateral to the NGF infusion (TrkA: Fig. 7C, D; $p75^{NTR}$: Fig. 7E, F). Apparent differences between background labeling in the NGF- and vehicle-infused hemispheres for Fig. 7B–F are probably due to increased immunore-

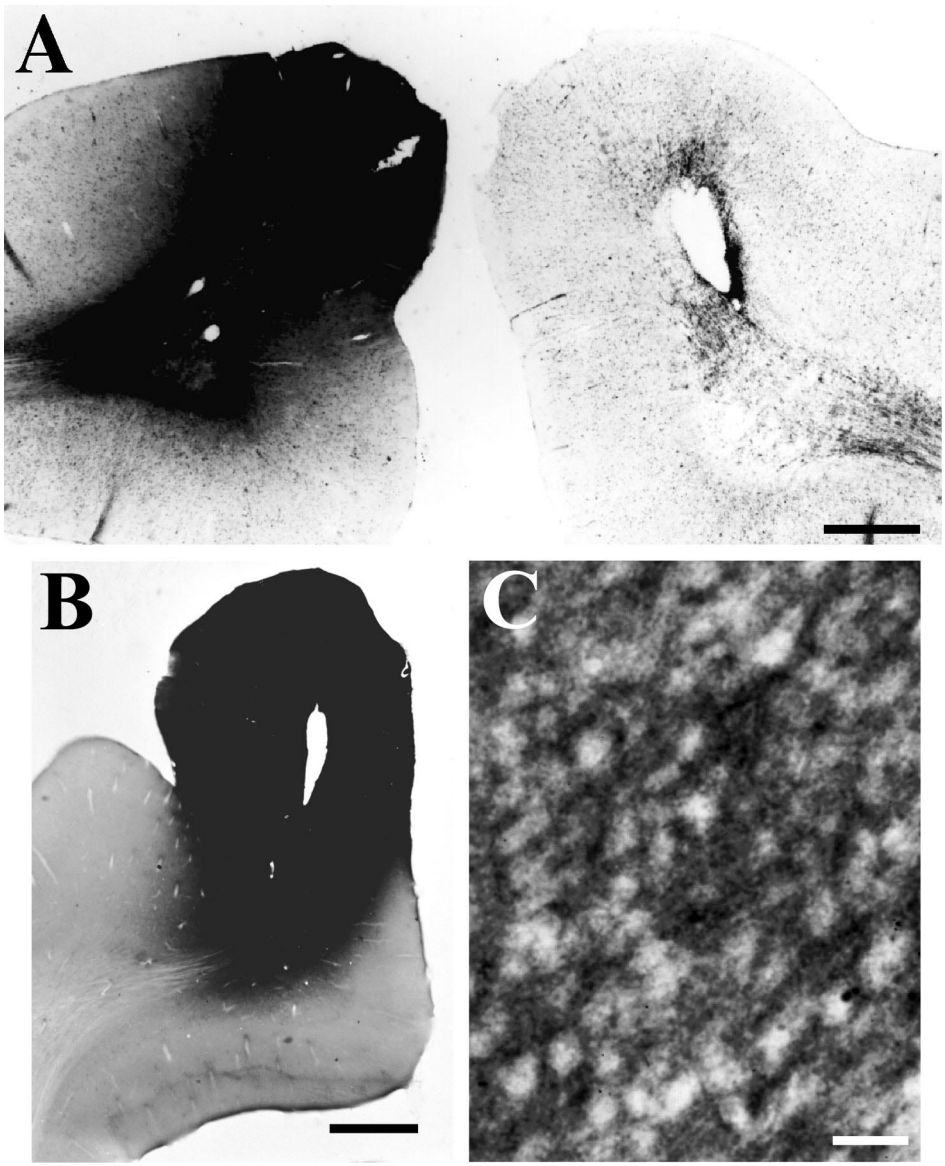

Fig. 6. NGF delivered to primary visual cortex with osmotic minipumps is detectable over a large volume of tissue. Infusion of NGF was demonstrated with DAB immunohistochemistry using an anti-NGF antibody. (A) The NGF-infused hemisphere is on the left and the vehicle-infused hemisphere on the right. The spread of detectable NGF is a few millimeters from the infusion site. This animal received NGF for 4 days prior to perfusion. (B) Another hemisphere infused with NGF for 12 days. (C) Higher power view of the infusion site. Exogenously applied NGF is found primarily in the cortical neuropil and seems to avoid cortical neuronal cell bodies. Scale bars = 1 mm (A, B) and 25 μm (C).

activity in processes of basal forebrain cholinergic neurons as a result of cortical NGF infusion. These differences were not observed outside the basal forebrain region in the same tissue sections (data not shown). In any case, labeled basal forebrain cell bodies were large and easily discriminable from the neuropil.

Labeling of $p75^{NTR}$-, TrkA-, or ChAT-like immunoreactivity on fibers in the cortex was not quantitatively compared in the two hemispheres, but Garofalo et al. (1992) did not observe any changes in cholinergic fiber length or number or size of cholinergic varicosities in cortex following intraventricular infusion of NGF in the adult rat. In conclusion, for all four proteins (NGF, TrkA, $p75^{NTR}$, and ChAT), the numbers of neurons in the septal nuclei, diagonal band of Broca, and substantia innominata that were positive for these markers were higher in the hemisphere ipsilateral to the NGF minipump than the contralateral hemisphere.

We verified the findings evident in the pictures of the immunostained sections by making blind counts of the numbers of positive cells in the two hemispheres. A total of 46 962 basal forebrain neurons in 70 sections from three animals were counted. The basal forebrain neurons located within the septal nuclei, diagonal band of Broca, and substantia innominata were counted separately, and the results are presented in Fig. 8. In animal K11, the differences between the two hemispheres were highly significant (TrkA: $P < 0.001$; $p75^{NTR}$: $P < 0.005$; ChAT

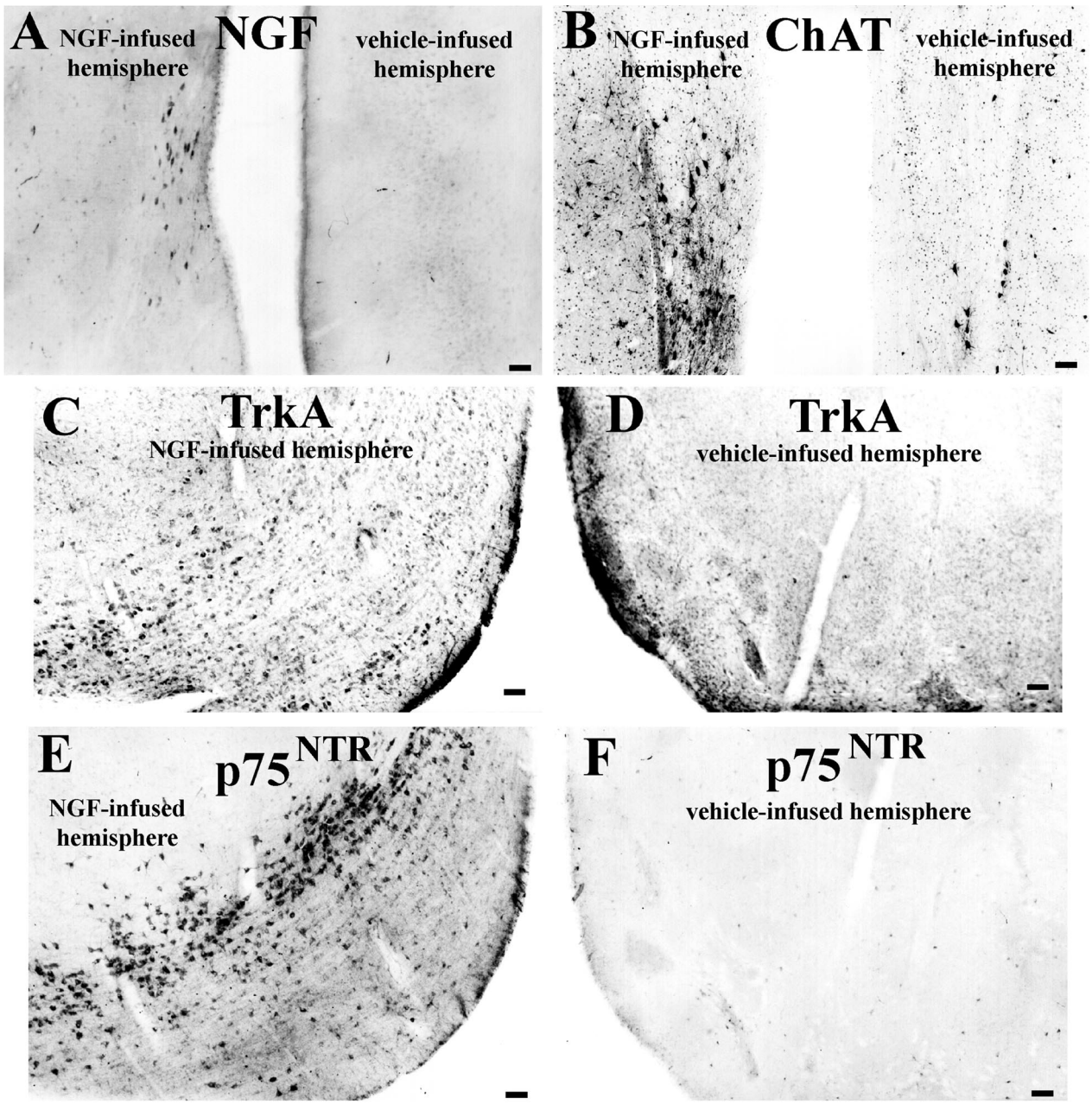

Fig. 7. Local cortical delivery of NGF increases NGF-, TrkA-, $p75^{NTR}$-, and ChAT-like immunoreactivity in basal forebrain neurons ipsilateral to the NGF-infused hemisphere. NGF-infused hemispheres are on the left, and vehicle-infused hemispheres are on the right. (A) NGF, septal nuclei, animal K11; (B) ChAT, septal nuclei, animal K93; (C, D) TrkA, substantia innominata and diagonal band of Broca, animal K11; (E, F) $p75^{NTR}$, diagonal band of Broca, animal K11. Scale bar = 100 μm.

not tested). In animal K93, the differences were smaller but reached significance for $p75^{NTR}$ ($P < 0.05$) and ChAT ($P < 0.05$), although not for TrkA. Finally, in the third animal (K108), similar numbers of TrkA-, $p75^{NTR}$-, and ChAT-positive neurons were labeled on the two sides. Possible explanations for the interanimal variability in these experiments are presented in the Discussion. However, we tested whether the volume of NGF infusion in cortex correlated with the magnitude of the hemispheric differences and found no relationship between these two measures (data not shown).

DISCUSSION

We have shown that local cortical infusion of NGF affects basal forebrain cholinergic neurons on the same side of the brain, increasing immunoreactivity for NGF itself, the low-affinity NGF receptor $p75^{NTR}$, the high-affinity NGF receptor TrkA, and the cholinergic marker ChAT. However, cortical NGF administration had no effect on the normal segregation of geniculocortical afferents into ocular dominance columns or on physiological measures of ocular dominance plasticity induced by MD.

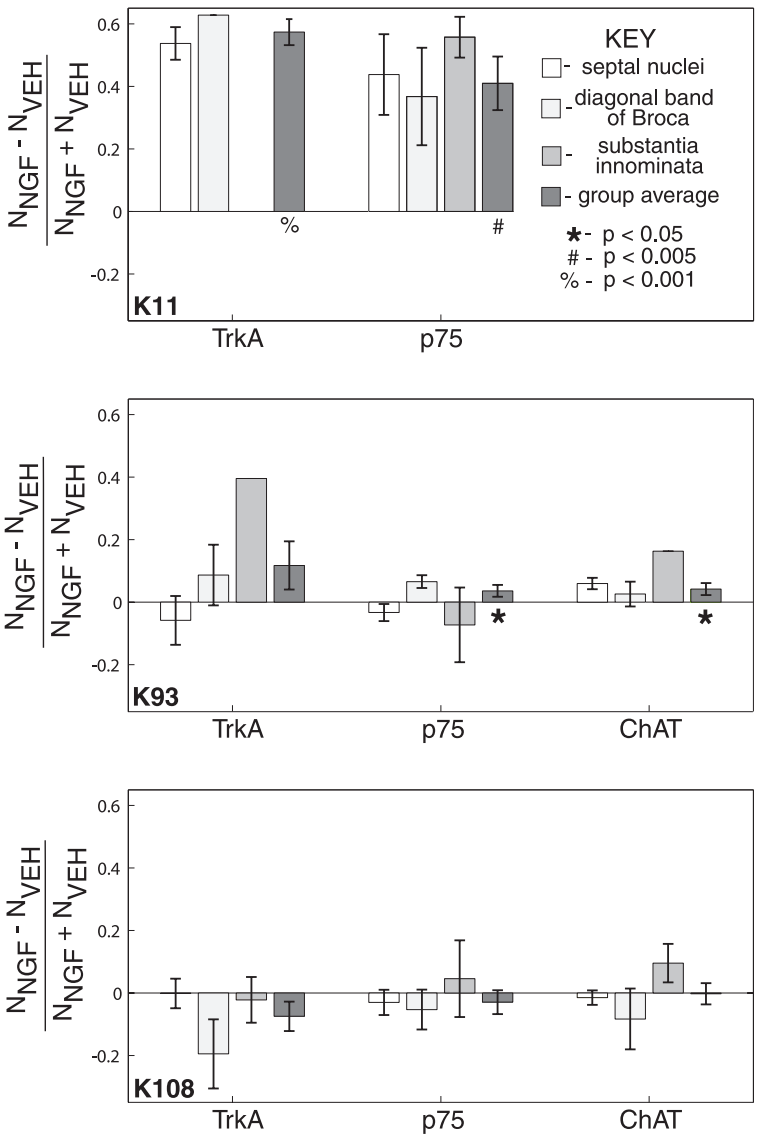

Fig. 8. Quantification of hemispheric differences in TrkA, $p75^{NTR}$, and ChAT expression in basal forebrain following NGF infusion. For each section, numbers of positive neurons for each antigen were compared in the two hemispheres using a contrast index (see Experimental procedures). Average contrast indices were computed for each of three basal forebrain regions (septal nuclei, diagonal band of Broca, and substantia innominata) that project to the cerebral cortex in cats. Statistical tests were performed for averages of the three brain regions that were weighted based on the total number of positive neurons per section. Error bars indicate weighted S.E.M.

Comparison with intraventricular infusion of NGF

ICV administration of NGF has previously been shown to cause a variety of changes in basal forebrain cholinergic neurons. Enzymatic activity of ChAT is elevated following NGF treatment in neonatal (Gnahn et al., 1983) and adult (Fusco et al., 1989) rats. In addition, ICV delivery of NGF results in an increase in the size of basal forebrain neurons in developing (Li et al., 1995) and adult rats (Higgins et al., 1989), and this hypertrophy was also observed following intracortical NGF infusion (Hu et al., 1997). Some of the increases in protein levels in basal forebrain neurons we have observed after local cortical delivery of NGF also occur with ICV administration (NGF: Yan et al., 1994, $p75^{NTR}$: Fusco et al., 1991). However, protein levels of TrkA have been reported to be unaffected by ICV injections of NGF in the rat (Li et al., 1995). Overexpression of NGF under the control of the glial fibrillary acidic protein promoter increased the number of ChAT-positive neurons in the medial septal nucleus (Kawaja et al., 1998). These methods result in a widespread distribution of NGF in the brain (overexpression: Kawaja and Crutcher, 1997; ICV injections: Yan et al., 1994), and it is not known whether the effects of NGF in these studies are mediated by signaling through axons, dendrites, or cell bodies of basal forebrain neurons. Thus, to our knowledge, the experiments reported here represent the first evidence that NGF delivered locally to the axons of basal forebrain neurons can increase immunoreactivity for NGF, $p75^{NTR}$, TrkA, and ChAT in the cell bodies of these neurons through retrograde signaling.

Increased protein expression or retrograde transport?

It is unclear from these data whether the increases in NGF-, TrkA-, and $p75^{NTR}$-like immunoreactivity in the basal forebrain are due to increased retrograde transport of these proteins from the cortex or to increased synthesis in basal forebrain neurons themselves. There is evidence in the literature for both possibilities. Retrograde transport of radiolabeled NGF to basal forebrain following cortical administration has been demonstrated for the adult (Seiler and Schwab, 1984) and developing (Domenici et al., 1994b) rat. In addition, transection of the septohippocampal projection results in accumulation of $p75^{NTR}$- (Johnson et al., 1987) and TrkA- (Loy et al., 1994) like immunoreactivity on the distal side of the lesion, suggesting that these proteins can be retrogradely transported in septohippocampal axons. Finally, cortical administration of antibodies against either $p75^{NTR}$ (Taniuchi et al., 1986) or TrkA (Pizzorusso et al., 1999) results in retrograde transport of the antibodies to cells in the basal forebrain. It seems unlikely that the NGF-induced increase in NGF-like immunoreactivity in basal forebrain neurons is due to synthesis of NGF by these neurons, since almost all of the basal forebrain neurons that express NGF receptors are ChAT-positive ($p75^{NTR}$: Dawbarn et al., 1988; Batchelor et al., 1989; TrkA: Sobreviela et al., 1994), while none of the basal forebrain neurons that express NGF mRNA are cholinergic (Lauterborn et al., 1995). However, intraventricular injections of NGF increase the expression of mRNA for $p75^{NTR}$ (Higgins et al., 1989), TrkA (Holtzman et al., 1992), and ChAT (Higgins et al., 1989) in basal forebrain neurons, and this could also lead to increased levels of protein expression in the cell bodies of these neurons.

Regardless of whether the increases in the number of neurons positive for TrkA, $p75^{NTR}$, and ChAT are due to retrograde transport or increased expression of these proteins in basal forebrain neurons projecting to the infusion site, it is likely that the population of neurons which responded to NGF were already expressing NGF receptors and ChAT at the beginning of the infusion. Assuming that the effects of NGF on protein expression are mediated by the NGF receptors TrkA and/or $p75^{NTR}$, neurons that do not express NGF receptors on their axon terminals would be unable to respond to exogenous NGF. Since those basal forebrain neurons that normally express NGF receptors almost always express ChAT as well ($p75^{NTR}$: Dawbarn et al., 1988; Batchelor et al., 1989; TrkA: Sobreviela et al., 1994), the increase in ChAT-positive neurons after cortical NGF infusion probably occurred in a population that was already expressing ChAT at lower levels prior to the NGF delivery.

Possible sources of variability in the magnitude of the effects of NGF on protein expression in the basal forebrain

In the data set of three animals presented in Fig. 8, one animal showed highly significant effects of NGF infusion (K11), one had smaller but statistically significant effects (K93), and one indicated no effect of NGF on expression of NGF, TrkA, p75NTR, and ChAT in the basal forebrain (K108). We believe that this variability is due to differences in the amount of basal labeling in the three animals. The number of positive cells in the basal forebrain of the vehicle-infused hemispheres varied substantially from animal to animal. This is probably due, in part, to the quality of perfusion and fixation of the brain in these animals. It is well known that the long periods of anesthesia required for single-unit electrophysiological and intrinsic signal optical imaging experiments result in a decrease in the quality of perfusion. For the first animal (K11), extensive imaging and single-unit recording were carried out for both hemispheres, and this animal had the fewest number of positive cells in the vehicle-infused hemisphere of the three animals (five out of five antibody/brain area combinations) and the largest effect of NGF infusion. Some images from both hemispheres and recording from only one hemisphere were obtained for K93, and it had intermediate levels of staining in the control hemisphere (five out of five antibody/brain area combinations) and an intermediate size of NGF effect. Finally, no recordings or imaging were performed for K108. This animal showed the highest levels of control hemisphere staining of the three animals (eight out of nine antibody/brain area combinations) and no effect of NGF.

Since the effects of NGF documented here are probably due to an increase in levels of TrkA, $p75^{NTR}$, and ChAT in neurons that were already expressing those proteins (see *Increased protein expression or retrograde transport?*), we propose that high levels of labeling of basal levels of these proteins obscured the effects of NGF. DAB immunohistochemical techniques rely on the production of an opaque precipitate in cells expressing the protein of interest, and this precludes precise measurement of the amount of label per cell. The results are typically quantified by setting a threshold and counting the number of cells above this threshold (the number of positive cells). If basal labeling is high, many cells will exceed this threshold in the control conditions, and effects of NGF will be occluded. In the extreme case, all cells expressing a protein of interest will be positive in the control hemispheres, and even if NGF infusion increases protein expression in all of these neurons, this increase will not be detectable. We believe that this ceiling effect artifactually decreased the measured effects of NGF in K108 and, to a lesser extent, in K93. The decreased quality of perfusion of K11 (and to a lesser extent, K93) decreased basal levels of positive cells and allowed the increase of positive neurons following NGF treatment to be more easily detected.

This explanation provides a plausible account for the interanimal variability observed in these experiments. It suggests that the negative results in K108 and the modest results in K93 are due to masking of the effects of NGF by high levels of basal labeling. Although this artifact could attenuate or completely occlude a real effect of NGF, it is very difficult to imagine how it would generate the large positive results seen in K11 or the significant hemispheric differences observed in K93. The measurements are relative comparisons between counts

from the two hemispheres within a single tissue section, so interanimal differences in perfusion or other factors involving the quality of the tissue cannot account for the measured hemispheric differences. In conclusion, we believe that the ceiling effects described above are likely to result in a substantial underestimate of the size of the effects of NGF, and we are unable to find an explanation in which an artifact could account for the significant hemispheric differences we have observed.

Possible functional consequences of NGF signaling in basal forebrain

The lack of effect of NGF on cortical development and plasticity observed in this study is in general agreement with the results of local cortical administration of NGF on ocular dominance plasticity in kittens (Galuske et al., 2000; Gillespie et al., 2000), on the later stages of ocular dominance column formation in kittens (Cabelli et al., 1995) and on the MD-induced shrinkage of cell bodies in the deprived eye laminae of ferret LGN (Riddle et al., 1995). ICV administration of NGF was somewhat more effective in the kitten, preventing the atrophy of deprived eye LGN neurons caused by MD (Carmignoto et al., 1993). In addition, infusion of NGF into the ventricle resulted in partial attenuation of both physiological ocular dominance plasticity (Carmignoto et al., 1993) and the loss of visual acuity in the deprived eye as assessed behaviorally (Fiorentini et al., 1995).

However, in the rat, NGF completely blocks the effects of MD, regardless of whether it is delivered intraventricularly (reviewed in Cellerino and Maffei, 1996) or intracortically (Lodovichi et al., 2000). The mechanisms underlying this apparent species difference in the efficacy of NGF remain unknown. Although we observed no effect of local cortical delivery of NGF on ocular dominance column development or plasticity, there was increased immunoreactivity for NGF, TrkA, $p75^{NTR}$, and ChAT in basal forebrain neurons projecting to the infusion site. We propose that differences between the rat and the cat in the size of cholinergic axon arbors in the cortex could account for differences in the efficacy of NGF in these two species.

Double label retrograde studies have indicated that basal forebrain cholinergic axon arbors in the cortex are approximately 1–1.5 mm wide in the rat (Price and Stern, 1983). In the cat, however, many single basal forebrain cholinergic neurons have axon arbors that cover the entire cerebral cortex. Adams et al. (1986) made injections of two different retrograde tracers in anterior and posterior cortical areas of adult cats. The distance between these injection sites was about 4 cm of cortical surface, corresponding to almost the entire anterior–posterior length of the cortex. Of those basal forebrain neurons retrogradely labeled by the posterior injection, 28% were also labeled by the tracer injected in the anterior area. This pattern was also observed for injections along a mediolateral axis. This anatomical pattern in the cat suggests that local cortical infusion of NGF will only weakly activate cholinergic neurons projecting to the infused area, because only a small part of each arbor will be exposed to NGF. On the other hand, the cortical delivery of NGF in the rat will strongly activate those cholinergic neurons projecting to the infused region, since relatively more of each arbor will be contained within the infused area. Experiments are currently underway to test whether local cortical infusion of NGF in the rat results in a larger up-regulation of protein expression in basal forebrain neurons than we have observed in the cat.

This species difference may also occur, albeit to a lesser extent, for intraventricular infusions. Since the cholinergic arbor widths in the rat are small, the spread of NGF following intraventricular administration only needs to cover a small area in order to maximally activate those cholinergic neurons projecting to that area. In the cat, however, NGF would have to diffuse practically over the entire brain in order to reach all of the axon branches of a given basal forebrain cholinergic arbor. In addition, this model accounts for the larger effects observed in the cat with intraventricular NGF infusion (Carmignoto et al., 1993; Fiorentini et al., 1995) than with intracortical infusion (Cabelli et al., 1995; Galuske et al., 2000; Gillespie et al., 2000; present study). Intraventricular infusion of NGF will result in a much larger spread of NGF than intracortical infusions, thereby stimulating a greater fraction of the arbors of individual cholinergic axons. It is also possible that intraventricular but not intracortical delivery allows NGF to diffuse into the basal forebrain. A direct action of NGF on basal forebrain cell bodies might also contribute to the greater efficacy of NGF on visual cortical plasticity following intraventricular administration.

It is interesting to note that a developmental shift of TrkA protein expression from basal forebrain cholinergic cell bodies to axons has been reported in the rat (Li et al., 1995). If this also occurs in the cat, local cortical infusion of NGF should be more effective in the adult than in the kitten. This is indeed the case, as cortical delivery of NGF in the adult cat allows reverse ocular dominance plasticity to occur at an age at which it normally would not (Gu et al., 1994; Galuske et al., 2000).

We hypothesize that if enough of the axon arbor of a given cholinergic neuron is exposed to NGF (as in the rat experiments), the neuron will not only increase protein expression as observed in this study but will also release more acetylcholine in the region of cortex that was infused with NGF. There is substantial evidence that basal forebrain neurons respond to exogenous NGF by increasing their activity and acetylcholine release. One week of intraventricular infusion of NGF in adult rats resulted in an increase in cortical extracellular acetylcholine as measured with microdialysis (Maysinger et al., 1992). Additionally, neurons in grafts of fetal rat septal tissue into the eye (thereby allowing NGF to be administered to these neurons without interference from the blood–brain barrier) acutely increase their firing rate after NGF administration, and this effect was not observed for hippocampal or spinal cord grafts (Palmer et al., 1993). Finally, visual cortical synaptosomes prepared from rats at the height of the critical period for ocular dominance plasticity exhibited

increased depolarization-evoked acetylcholine release following NGF administration (Sala et al., 1998).

Increased acetylcholine release in the cortex is likely to increase the activity of cortical neurons. When acetylcholine or muscarinic agonists were delivered iontophoretically to visual cortex, the most common and consistent effect on sensory responses was a facilitation of suprathreshold responses (Sillito and Kemp 1983; Sato et al., 1987). In addition, the pairing of iontophoresis of acetylcholine with visual stimulation that was not optimal for either stimulus orientation, direction, or eye of preference caused potentiation of responses to these nonoptimal stimuli (Greuel et al., 1988). Essentially all of the acetylcholine in cat visual cortex comes from basal forebrain cholinergic terminals (Bear et al., 1985).

Thus, if exogenously delivered NGF increases cholinergic function in the visual cortex by activating cortically projecting basal forebrain neurons, this could increase the size of responses evoked by natural visual stimulation through the sutured eye during a period of MD. Perhaps deprived eye responses are potentiated to a point at which postsynaptic cortical neurons no longer effectively discriminate between sets of inputs representing the two eyes, thereby reducing or preventing ocular dominance plasticity. This hypothesis is supported by the results of Caleo et al. (1999), in which NGF was unable to block the plasticity induced by intraocular administration of tetrodotoxin (TTX). In this case, because there was little or no activity in deprived eye visual inputs to the cortex, potentiation of deprived eye activity by acetylcholine was not possible. Although this model predicts that cortical infusion of NGF should more powerfully activate basal forebrain neurons in the adult cat than in the kitten, it does not account for the paradoxical shift in binocular responses towards the deprived eye that has been observed in these studies (Gu et al., 1994; Galuske et al., 2000).

Any facilitation of cholinergic inputs to cortex by NGF administration is not likely to be a result of sprouting of cholinergic axons in the rat visual cortex, as intraventricular injections of NGF had no effect on cortical ChAT activity (Domenici et al., 1991), cortical acetylcholinesterase distribution patterns (Berardi et al., 1993), or cholinergic fiber length or number or size of cholinergic varicosities in cortex (Garofalo et al., 1992). We also did not observe any evidence of cholinergic fiber sprouting in the NGF-infused area in visual cortex (data not shown). Although prolonged treatment with NGF has no effect on spontaneous activity, visual responsiveness, or selectivity for stimulus orientation or spatial frequency in anesthetized rats or kittens (Domenici et al., 1992; Carmignoto et al., 1993; Lodovichi et al., 2000), these results do not argue against a facilitation of cholinergic input to the cortex, as activity in the basal forebrain cholinergic system is strongly related to the behavioral state of the animal (Détári and Vanderwolf, 1987), and general anesthetics decrease acetylcholine release in the cortex (Pepeu, 1974). It is therefore unlikely that effects on cortical responsiveness to sensory stimulation as a result of enhanced cholinergic function would be observed in recordings from anesthetized animals.

Possible actions of NGF on cortical neurons

A number of studies are consistent with the possibility of exogenous NGF acting directly on cortical neurons. NGF increased glutamate release from synaptosomes prepared from rat visual cortex, and this effect was not blocked by nicotinic or muscarinic cholinergic antagonists, suggesting that it is not due to modulation involving cholinergic afferents (Sala et al., 1998). In addition, incubation of slices of visual cortex from developing ferrets in NGF-containing medium for 36 h resulted in changes in dendritic morphology of cortical neurons (McAllister et al., 1995). There have been some reports of trkA mRNA expression in cortex using *in situ* hybridization (Miranda et al., 1993), northern blots (Valenzuela et al., 1993), and polymerase chain reaction, PCR (Cellerino and Maffei, 1996). However, other studies have not replicated these results (Holtzman et al., 1992; Merlio et al., 1992; Ringstedt et al., 1993).

Our results are not consistent with a direct action of NGF on cortical neurons. Immunostaining for NGF within the infusion site showed that exogenous NGF was confined mainly to the cortical neuropil and seemed to avoid cortical neuronal cell bodies. We did not observe any TrkA immunoreactivity in cortical neurons (data not shown), and this is in agreement with other studies (Sobreviela et al., 1994; Holtzman et al., 1995). In addition, Domenici et al. (1994b) found that the number of basal forebrain neurons labeled by a local injection of radiolabeled NGF into rat occipital cortex was one to two orders of magnitude greater than the number of labeled cortical neurons near the injection site, depending on the age of the animal.

Alzheimer's disease and retrograde transport

Basal forebrain neurons in patients with Alzheimer's disease have decreased amounts of trkA mRNA (Mufson et al., 1996) and TrkA-like immunoreactivity (Salehi et al., 1996), and TrkA protein levels are lower in the cerebral cortex of these patients (Mufson et al., 1997). In addition, NGF levels are increased in cerebral cortex and decreased in nucleus basalis in Alzheimer's disease (Scott et al., 1995), suggesting that this disease could be associated with impaired TrkA-dependent retrograde transport of NGF. It is possible that the decreases in TrkA expression and retrograde transport are simply consequences of other processes that are responsible for the etiology of Alzheimer's disease. However, if Alzheimer's disease is primarily due to a decreased ability of basal forebrain neurons to obtain trophic support (Hefti, 1983; Hefti and Weiner, 1986), treatments resulting in an increase of TrkA or $p75^{NTR}$ expression may be useful. We have shown that delivery of NGF to the axon terminals of basal forebrain neurons increased TrkA- and $p75^{NTR}$-like immunoreactivity in the cell bodies of these neurons. However, these changes in protein expression did not result in alterations of cortical development or plasticity. If the hypothesis presented above concerning species differences in the sizes of cholinergic axon arbors is correct, our results indicate that a more com-

plete description of the anatomical organization of the basal forebrain cholinergic system in humans could be critical in the design of therapeutic interventions in Alzheimer's disease.

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