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Neurotrophins Regulate Dendritic Growth in Developing Visual Cortex

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Summary

Although dendritic growth and differentiation are critical for the proper development and function of neocortex, the molecular signals that regulate these processes are largely unknown. The potential role of neurotrophins was tested by treating slices of developing visual cortex with NGF, BDNF, NT-3, or NT-4 and by subsequently visualizing the dendrites of pyramidal neurons using particle-mediated gene transfer. Specific neurotrophins increased the length and complexity of dendrites of defined cell populations. Basal dendrites of neurons in each cortical layer responded most strongly to a single neurotrophin: neurons in layer 4 to BDNF and neurons in layers 5 and 6 to NT-4. In contrast, apical dendrites responded to a range of neurotrophins. On both apical and basal dendrites, the effects of the TrkB receptor ligands, BDNF and NT-4, were distinct. The spectrum of neurotrophic actions and the laminar specificity of these actions implicate endogenous neurotrophins as regulatory signals in the development of specific dendritic patterns in mammalian neocortex.

Introduction

Each of the six layers of mammalian cerebral cortex contains a unique assembly of neuronal types with characteristic axonal projections, physiological properties, and dendritic arbors. As dendrites are the site of most synapses, dendritic development determines the number and pattern of synapses received by each neuron (Hume and Purves, 1981; Purves and Hume, 1981; Purves et al., 1986). Pyramidal neurons, the primary excitatory neurons in cortex, undergo extensive dendritic growth and differentiation soon after completing migration, leading to the specific apical and basal dendritic arborizations of mature cortical neurons (Ramon y Cajal, 1929; Eayrs and Goodhead, 1959; Wise et al., 1979; Miller and Peters, 1981). The final form and extent of dendritic arbors result from interactions between intrinsic developmental programs (Banker and Cowan, 1979; Bartlett and Banker, 1984) and local environmental cues, including levels and patterns of activity (Valverde, 1968; Harris and Woolsey, 1981; Katz and Constantine-Paton, 1988; Katz et al., 1989; Bailey and Kandel, 1993). However, the molecular signals mediating these interactions in neocortex remain unknown.

Neurotrophins are attractive candidates for such signals, as one member of this family of factors, nerve growth factor (NGF), strongly influences dendritic complexity in the peripheral nervous system (PNS) in vivo (Purves et al., 1988; Snider, 1988; Ruit et al., 1990; Ruit and Snider, 1991). The neurotrophins comprise at least four structurally related proteins: NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/neurotrophin-5 (NT-4) (for review, see Lindsay et al., 1994; NT-6 has recently been cloned in fish [Gotz et al., 1994]). Also, neurotrophins exert their effects through activation of members of the Trk family of tyrosine kinase receptors (for review, see Chao, 1992). These factors and their receptors are expressed in discrete layers of neocortex during the time dendrites develop (Allendoerfer, et al., 1990; Merlio et al., 1992; Cabelli et al., 1993, Soc. Neurosci., abstract; Ringstedt et al., 1993; Lindsay et al., 1994). However, assigning specific biological functions to neurotrophins has been hampered by the difficulty of manipulating the cortical environment in vivo and by the loss of laminar identity and cell polarity in dissociated tissue culture.

We examined whether the four neurotrophins modulated dendritic growth in short-term organotypic slice cultures of developing ferret visual cortex. Organotypic slices preserve the local three-dimensional environment of each neuron, the laminar organization of cortex, and the pattern of connections within and between these layers (Bolz et al., 1990; Molnar and Blakemore, 1991; Stoppini et al., 1991; Yamamoto et al., 1992; Roberts et al., 1993). We found that the growth and complexity of cortical dendrites were regulated by neurotrophin treatment. Neurons in each cortical layer responded to subsets of neurotrophins with distinct effects on basal and apical dendrites. Within a single cortical layer, each neurotrophin, including the two TrkB ligands, elicited a unique pattern of dendritic changes.

Results

The six layers of mammalian cerebral cortex define morphological and functional groups of neurons. These layers differ in their expression of neurotrophins and Trk receptors. If dendritic growth and differentiation are regulated by neurotrophins, then dendritic responses to each neurotrophin should differ across cortical layers. We therefore investigated whether all neurotrophins were equally effective in every cortical layer, which would imply that they cause some relatively nonspecific enhancement of dendritic growth, or whether certain neurotrophins were particularly effective in specific layers.

Coronal slices from the visual cortex of ferrets were prepared at postnatal day 14 (P14), at which time neurons in all of the cortical layers are rapidly elaborating dendrites. These slices were treated with NGF, BDNF, NT-3, or NT-4 for 36 hr in vitro. The detailed morphology of dendritic arbors of pyramidal neurons was visualized (Figure 1) using a particle-mediated gene transfer technique and subsequent immunostaining for β-galactosidase (Lo et al., 1994; also see Arnold et al., 1994). For each neurotrophin treatment, 40–50 cells in cortical layers 4–6 were reconstructed in detail; layers 1–3 were excluded from this

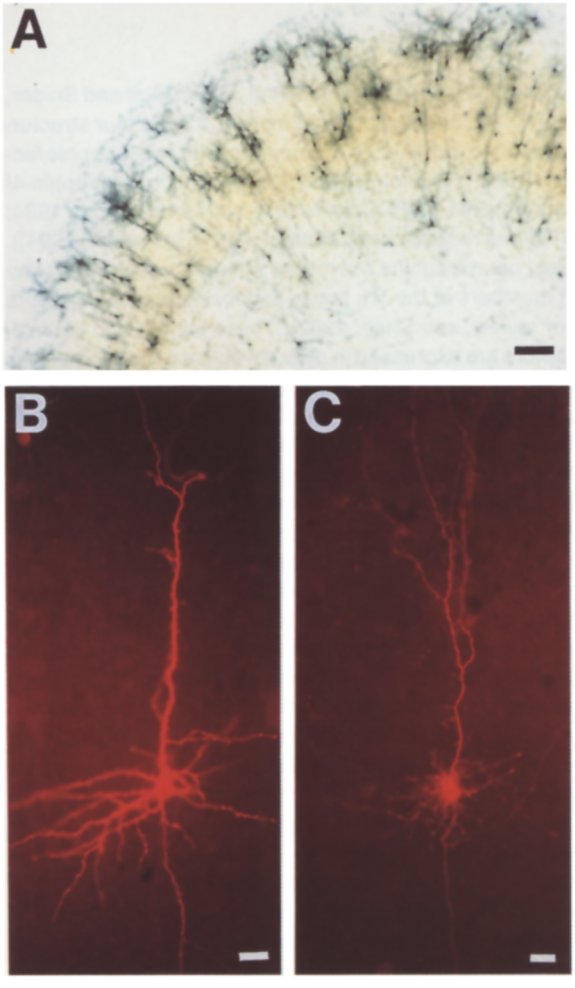

Figure 1. Pyramidal Neurons in Organotypic Slices of P14 Ferret Visual Cortex Transfected with a *lacZ* Expression Construct

(A) Field of transfected neurons cultured for 36 hr in the presence of NT-4 and visualized with X-Gal histochemistry. The number of neurons transfected in this section of a slice illustrates the level of transfection achieved by biolistics. Bar, 100 μm.

(B) Layer 5 pyramidal neuron from a slice treated with BDNF and immunostained for β-galactosidase. Bar, 10 μm.

(C) Layer 5 pyramidal neuron from a slice treated with NT-4. Note the halo of neurites extending from the cell body. Bar, 10 μm.

study, as layer 1 neurons are primarily nonpyramidal and layer 2/3 neurons have not yet migrated to their final position in the P14 ferret cortex (McConnell, 1988; Jackson et al., 1989). From this set of 680 cells (from at least 25 slices and 10 animals per treatment), four parameters of dendritic growth were measured directly (Table 1): the total length of dendrites and the numbers of primary dendrites, dendritic branches, and protospines.

Even a qualitative comparison of dendrites treated with the four neurotrophins indicated that these factors elicited marked changes in the dendritic organization of pyramidal neurons (Figure 2; also see Figures 3–5). In general, neurotrophin-responsive neurons had substantially longer and more complex arborizations. However, the effects of each neurotrophin on different cell populations varied widely (see Table 1).

To compare more easily the direction and magnitude of changes induced by each neurotrophin, we used a simple, weighted sum of the four measures of dendritic complexity. This measure, which we termed the dendrite modification index (DMI; see Experimental Procedures) was calculated from the data in Table 1, with the greatest weight given to changes in total dendritic length and the least weight to changes in the number of protospines. A DMI of 1 indicates no significant change in dendritic form from control values, while a DMI of 2 reflects dramatic changes. The DMI values for apical and basal dendrites are shown in Table 2; these values were used to calculate the percentage changes in DMI shown in Figure 6. In practice, the DMI values fit well with our qualitative impressions of the strength of effects of a given neurotrophin on dendritic organization.

The results of these investigations are presented in three parts. First, we demonstrate that basal dendrites of cells in each of the cortical layers responded most strongly to a single neurotrophin, with the other neurotrophins exhibiting considerably less or no influence. Second, we show that apical dendrites of neurons in each layer responded to a wider array of neurotrophins, suggesting that different compartments of the pyramidal cell that are functionally distinct—the basal and apical dendritic trees—respond differentially to the neurotrophins. Finally, we present evidence that the method used to visualize neurons, biolistic transfection, did not compromise the health of neurons.

Basal Dendrites in Each Layer Respond Most Strongly to a Single Neurotrophin

Basal dendrites of neurons in each layer were maximally enhanced by a single factor: layer 4 neurons by BDNF and layer 5 and layer 6 neurons by NT-4. These two factors, both TrkB ligands, were consistently the most effective modulators of dendritic form in all cortical layers.

Basal Dendrites of Layer 4 Neurons Respond Maximally to BDNF

Even prior to formal statistical analysis, the powerful effect of BDNF on dendrites of pyramidal cells in layer 4 was evident (see Figures 2 and 3). Basal dendrites were longer, more numerous, more highly branched, and covered with far more dendritic spines than either control cells or cells treated with the other three neurotrophins. Detailed measurements of these parameters (see Table 1) substantiated this impression. All four parameters of dendritic growth were almost doubled in BDNF-treated cells relative to untreated cells. The DMI of BDNF-treated cells was thus increased by almost 90% over controls (see Figure 6; Table 2). In contrast, the other neurotrophins had only modest effects, mostly increases in the number of protospines. The effects of NT-4 and NGF were similar—both enhanced the DMI by approximately 25%—and NT-3 had barely any effect (see Figure 6; Tables 1 and 2). Notably, though both BDNF and NT-4 exert their effects through TrkB receptors (Chao, 1992; Lindsay et al., 1994), their

Table 1. Mean Values for Four Measures of Dendritic Growth for Pyramidal Neurons in Untreated Slices and in Slices Treated with the Four Neurotrophins

	Layer 4					Layer 5					Layer 6				
	UT (62)	NGF (51)	NT-3 (50)	BDNF (52)	NT-4 (51)	UT (34)	NGF (28)	NT-3 (29)	BDNF (24)	NT-4 (28)	UT (55)	NGF (41)	NT-3 (40)	BDNF (42)	NT-4 (39)
Basal dendrites															
Dendritic length ($\mu m \times 10^{-1}$)	18.0 ± 1.4	24.8 ± 2.2	22.5 ± 3.0	34.0 ± 2.9	20.6 ± 2.2	74.6 ± 8.5	115.3 ± 10.6	87.4 ± 11.1	108.7 ± 10.1	124.5 ± 9.9	20.5 ± 1.6	18.3 ± 1.9	29.8 ± 4.7	16.8 ± 2.5	29.8 ± 3.1
Number of primary dendrites	3.2 ± 0.2	3.3 ± 0.3	3.6 ± 0.3	5.0 ± 0.4	5.2 ± 0.4	5.3 ± 0.4	6.0 ± 0.4	6.5 ± 0.6	5.5 ± 0.4	11.3 ± 0.7	3.6 ± 0.2	2.5 ± 0.2	3.4 ± 0.3	2.7 ± 0.2	5.9 ± 0.6
Number of branches	2.8 ± 0.3	3.3 ± 0.5	3.6 ± 0.5	6.4 ± 0.6	3.7 ± 0.6	12.1 ± 1.5	16.9 ± 1.9	12.7 ± 1.5	20.1 ± 1.9	24.5 ± 1.9	2.8 ± 0.4	2.0 ± 0.2	3.5 ± 0.6	1.7 ± 0.4	5.3 ± 0.8
Number of protospines	16.7 ± 1.3	28.6 ± 2.5	25.9 ± 2.0	34.4 ± 2.8	26.3 ± 2.4	56.2 ± 6.6	69.0 ± 7.9	66.0 ± 10.6	81.3 ± 8.0	115.1 ± 9.1	11.1 ± 1.0	12.2 ± 1.1	26.0 ± 3.7	10.0 ± 1.0	24.5 ± 3.0
Apical dendrites															
Dendritic length ($\mu m \times 10^{-1}$)	46.3 ± 2.2	67.3 ± 5.7	62.9 ± 3.8	99.9 ± 6.9	71.3 ± 4.1	115.5 ± 9.9	162.3 ± 14.3	126.4 ± 9.6	168.8 ± 14.3	142.5 ± 11.9	90.2 ± 3.5	108.2 ± 4.6	110.0 ± 5.5	106.7 ± 5.0	104.1 ± 5.0
Number of branches	9.9 ± 0.5	14.3 ± 0.9	13.9 ± 1.0	25.3 ± 1.9	22.0 ± 1.4	20.2 ± 2.0	28.9 ± 3.1	22.2 ± 2.3	34.6 ± 3.9	32.2 ± 2.9	11.4 ± 0.8	11.0 ± 0.7	15.6 ± 1.1	13.9 ± 1.1	16.3 ± 1.0
Number of protospines	29.4 ± 2.2	55.0 ± 3.3	54.9 ± 3.7	64.9 ± 5.2	108.8 ± 5.1	64.9 ± 6.8	80.6 ± 10.1	68.6 ± 10.9	85.4 ± 8.2	90.9 ± 5.5	33.9 ± 2.4	27.5 ± 2.0	61.7 ± 3.5	31.7 ± 2.1	48.4 ± 3.7

The mean (± 1 standard error) of (n) measurements of each parameter for each treatment in each cortical layer is shown. Statistical significance was calculated by single-factor ANOVA. Significance at the $p \leq .05$ level is indicated by a dashed box; $p \leq .001$, by a solid box. Single-factor ANOVA was also calculated between the different neurotrophins and confirmed the factor specificity described in the text. UT, untreated.

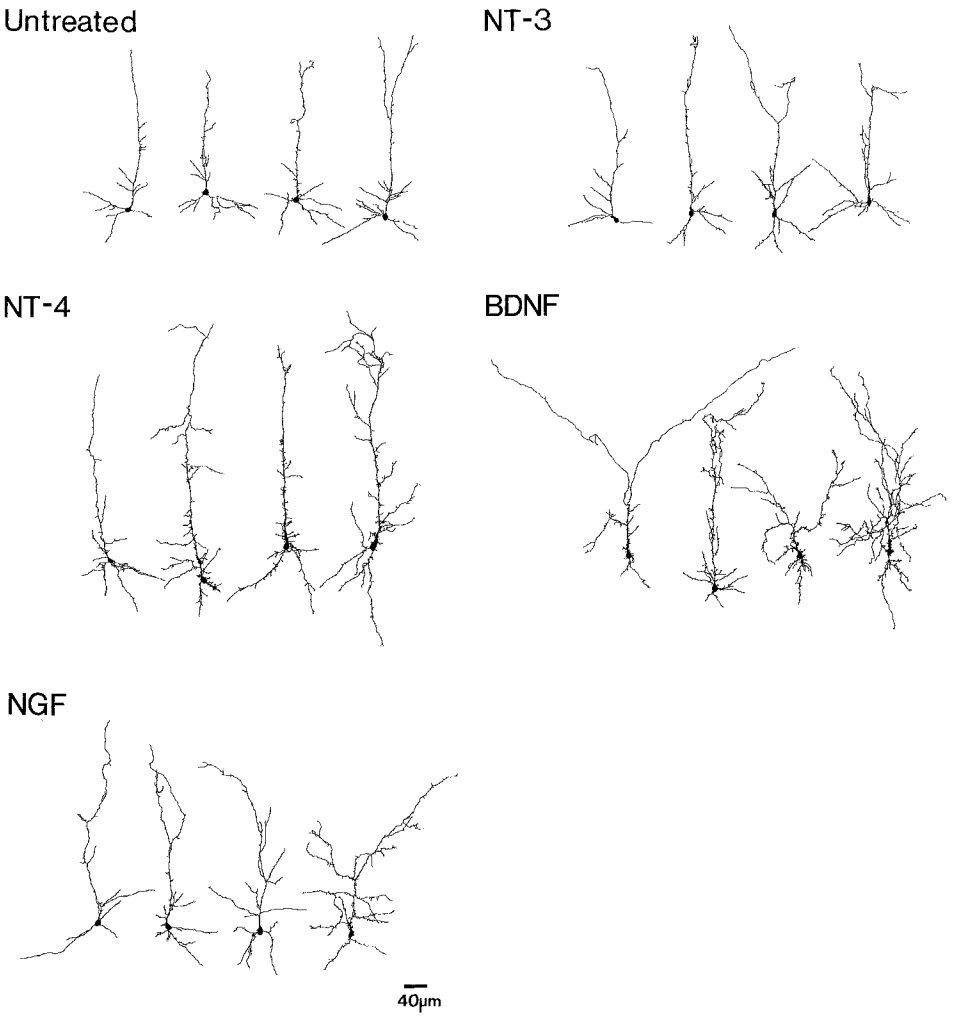

Figure 2. Representative Layer 4 Neurons from Neurotrophin-Treated Slices

The neurons depicted in each panel represent the range of morphologies across the sampled populations: the first 2 cells were selected to show dendrites less than or equal to the average complexity of arborization and the second 2 cells to show complexity greater than the average form. The complexity of basal and apical dendritic arborizations of neurons in layer 4 treated with BDNF was substantially increased. NT-4 and NGF treatment caused smaller increases in dendritic elaboration. In contrast, the effects of NT-3 in layer 4 were minimal and restricted to apical dendrites.

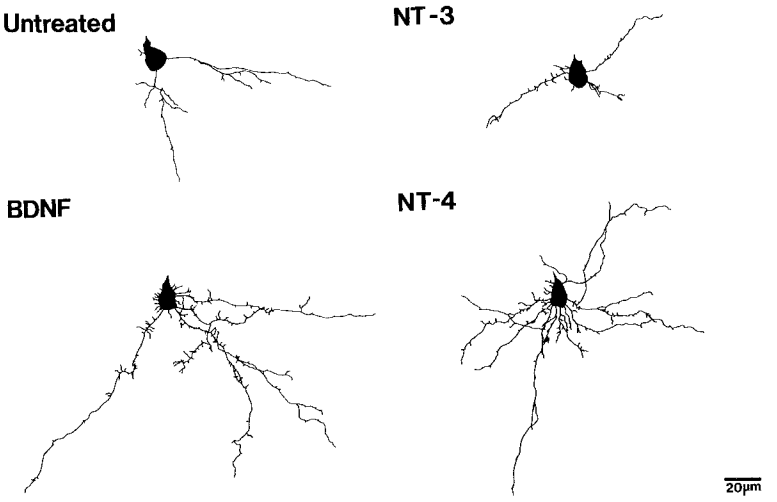

Figure 3. Fine Structure of Basal Dendrites of Layer 4 Neurons

Reconstructions of the basal dendrites and somas of layer 4 neurons illustrate the detailed morphology of control and neurotrophin-treated basal dendritic arbors. Note the marked increase in number and complexity of basal dendrites and their protospines with BDNF and NT-4 treatment.

Table 2. Values of the Dendrite Modification Index

	NGF	NT-3	BDNF	NT-4
Basal dendrites				
Layer 4	1.22	1.06	1.89	1.24
Layer 5	1.30	1.00	1.36	1.92
Layer 6	0.91	1.32	0.85	1.67
Apical dendrites				
Layer 4	1.49	1.43	2.32	2.03
Layer 5	1.38	1.00	1.52	1.29
Layer 6	1.08	1.34	1.09	1.29

The DMI was calculated from the data in Table 1 as described in Experimental Procedures. The DMI = 1.00 for cases in which treated values were not statistically distinct from untreated values, as determined by single-factor ANOVA (see Table 1). Values less than 1.00 indicate significant decreases in the DMI, while values greater than 1.00 indicate significant increases in the DMI.

effects on dendritic growth of layer 4 neurons were clearly distinct.

Basal Dendrites of Layer 5 Neurons Respond Maximally to NT-4

In contrast to neurons in layer 4, the basal dendritic arbors of pyramidal cells in layer 5 were greatly enhanced by treatment with NT-4. The response of layer 5 neurons to NT-4 was similar to the response of layer 4 neurons to BDNF: a conspicuous halo of more basal dendrites that were longer, more highly branched, and more densely covered with spines (Figure 4). NT-4 treatment increased the DMI by more than 90% (see Figure 6; Table 2). The effects of BDNF in this layer were much more modest (a 36% increase in DMI) and were comparable to the effects of

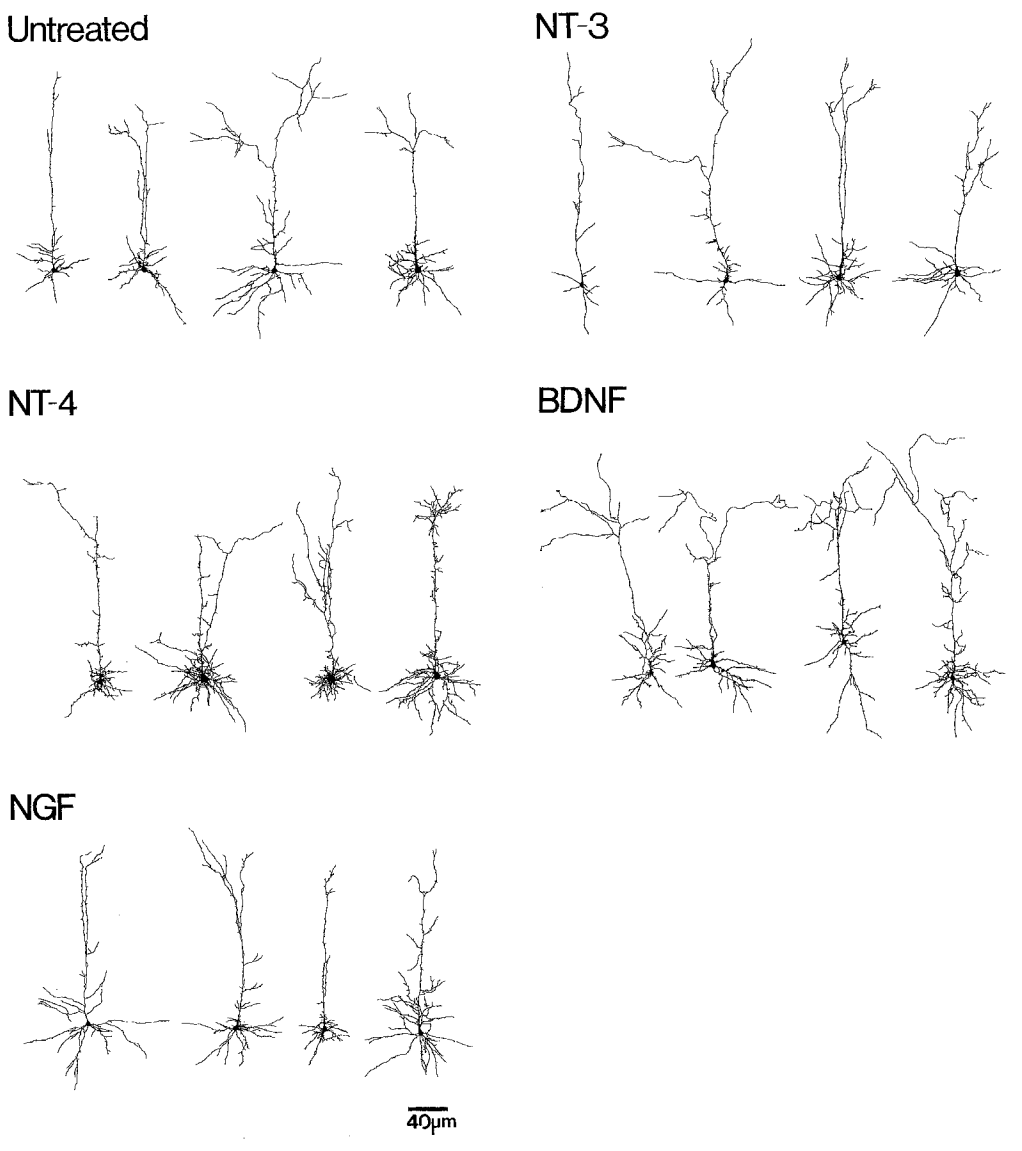

Figure 4. Representative Layer 5 Neurons from Neurotrophin-Treated Slices

The neurons depicted in each panel represent the range of morphologies across the sampled populations, as described in Figure 2. The effects of NT-4 on basal dendrites were particularly obvious: note the extensive halo of short neurites extending from the cell bodies. BDNF and NT-4 both produced large increases in apical dendritic arborizations. In contrast, NGF produced small enhancements of layer 5 dendrites; NT-3 had no effects in layer 5.

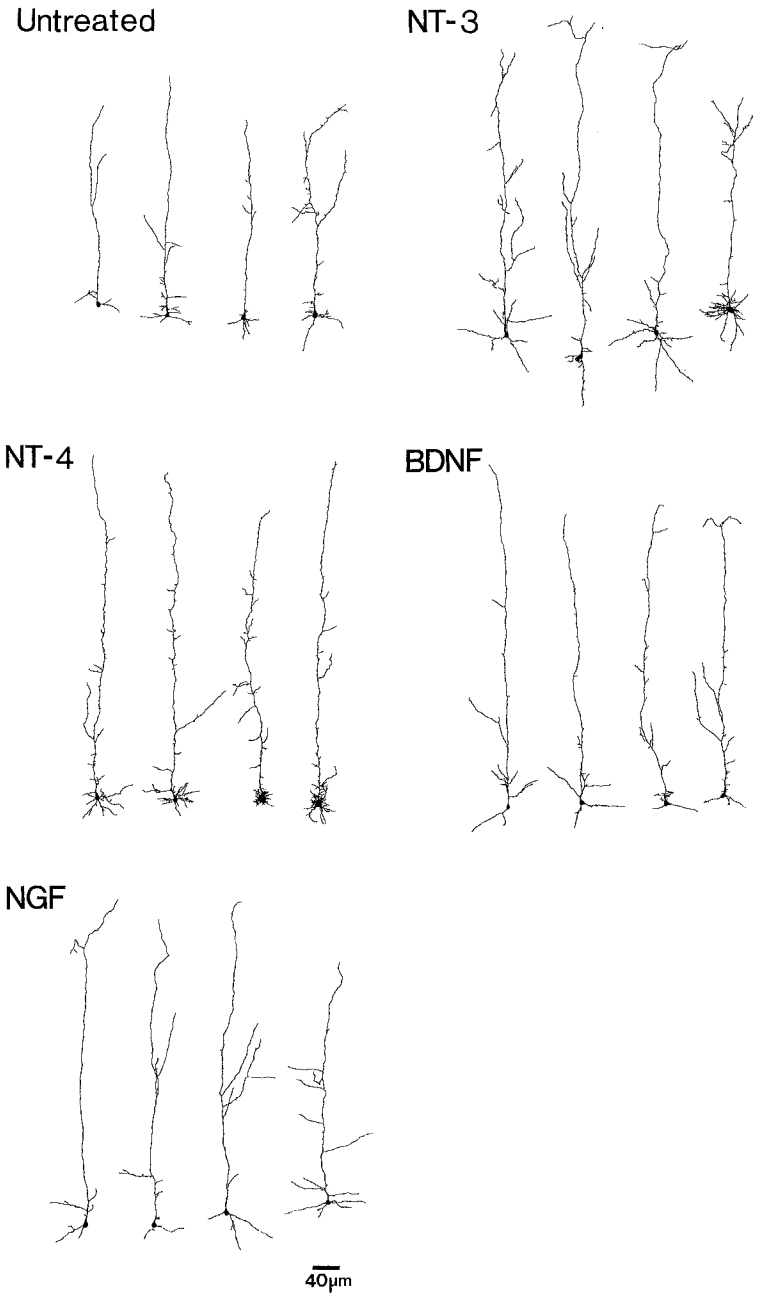

Figure 5. Representative Layer 6 Neurons from Neurotrophin-Treated Slices

The neurons depicted in each panel represent the range of morphologies across the sampled populations, as in Figures 2 and 4. Layer 6 neurons responded to NT-3 and NT-4 with increases in the amount and complexity of their dendrites. BDNF and NGF produced small decreases in basal dendritic arborization.

NT-4 and NGF in layer 4. As in layer 4, NGF modestly enhanced the DMI in layer 5 (a 30% increase due to small increases in total amount of dendrite), whereas NT-3 was completely ineffective (see Figure 6; Table 1).

Basal Dendrites of Layer 6 Neurons Respond to NT-4 and NT-3

As in layer 5, NT-4 had potent effects on basal dendrites of layer 6 pyramidal cells, resulting in a 68% increase in the DMI (Figure 5; see Figure 6; Tables 1 and 2). Layer 6 was the only layer in which NT-3, the TrkC ligand, significantly affected the DMI (a modest increase of roughly 30%), owing primarily to increases in overall dendritic length without increases in dendrite number or branching (Figure 5; see Figure 6; Tables 1 and 2).

Treatment of layer 6 neurons with BDNF and NGF produced an unexpected result. Rather than enhancing dendrites as in layers 4 and 5, BDNF and NGF decreased dendritic growth. BDNF decreased numbers and branching of basal dendrites, and NGF caused a similar, although less pronounced, decrease in the DMI (9%), owing to decreases in dendritic length and numbers of spines (Figure 5; see Figure 6; Tables 1 and 2).

Apical Dendrites of Pyramidal Cells Respond to a Broader Range of Neurotrophins than Basal Dendrites

Apical dendrites responded to a wider range of neurotrophins than their basal dendritic counterparts, but the

relative efficacy of different Trk receptor ligands was comparable. A slightly different version of the DMI was used (see Experimental Procedures) to summarize the overall effects of the different neurotrophins on apical dendrite elaboration.

Apical Dendrites of Layer 4 Neurons Respond Maximally to BDNF and NT-4

All four neurotrophins significantly increased the length, branching, and spines of apical dendrites in layer 4. As with basal dendrites, BDNF was most effective, more than doubling the DMI by increasing dendritic length, branching, and spines (see Figures 2 and 6; Tables 1 and 2). In fact, the largest dendritic response to any of the neurotrophins was the increase in layer 4 apical dendrites elicited by the TrkB ligands. The effects of NT-4 were only slightly smaller than those of BDNF, leading to a similar DMI. However, based on the effects on dendritic length alone (see Table 1), BDNF was clearly more potent than NT-4.

The effects of both TrkB ligands were greater than those of either NGF or NT-3 (Figure 6). If one simply ranks the extent to which the different neurotrophins modulated layer 4 apical dendrites using the DMI, the order is BDNF > NT-4 >> NGF = NT-3. For basal dendrites, the rank order was BDNF >> NT-4 = NGF > NT-3. Thus, the rank order of the effects of the different neurotrophins in layer 4 was roughly comparable for both basal and apical dendrites.

Apical Dendrites of Layer 5 Neurons Respond Primarily to BDNF and NT-4

Apical dendrites of layer 5 cells were most strongly enhanced by BDNF, which increased both their length and branching (see Figure 4; Table 1). This was in contrast to basal dendrites of the same cells, which were strongly enhanced by NT-4 and much less so by BDNF. NT-4 increased the number of apical dendritic branches and spines but not the overall amount of apical dendrite (see Figures 4 and 6; Table 1). In fact, the DMI was less for NT-4 than for NGF treatment (see Figure 6; Table 2). Apical dendrites of neurons in layer 5, like basal dendrites, did not respond to NT-3 (see Figure 6; Tables 1 and 2).

Unlike the conservation of rank order for neurotrophin effects on DMI in layer 4, the order of effects was different between apical and basal dendrites for layer 5 neurons. The DMI rank order for basal dendrites was NT-4 >> BDNF = NGF >> NT-3 versus BDNF > NGF > NT-4 >> NT-3 for apical dendrites (see Figure 6). The responses of apical dendrites to BDNF and NT-4 reinforced our conclusion, based on the responses of basal dendrites, that the actions of these two ligands were distinct.

Apical Dendrites of Layer 6 Neurons Respond Only Modestly to NT-3 and NT-4

In layer 6, neurons responded to NT-3 and NT-4 with modest increases in total apical dendrite length, number of branches, and number of spines (see Figures 5 and 6; Table 1). As we observed for basal dendrites, NGF and BDNF had only weak effects on apical dendrites (see Figures 5 and 6; Table 1). In general, the effects of neurotrophins on apical dendrites in layer 6 roughly paralleled those on basal dendrites in the same layer. However, the negative influence of BDNF was not observed, and NT-3 was as or more effective than NT-4 (see Figure 6; Tables 1 and 2).

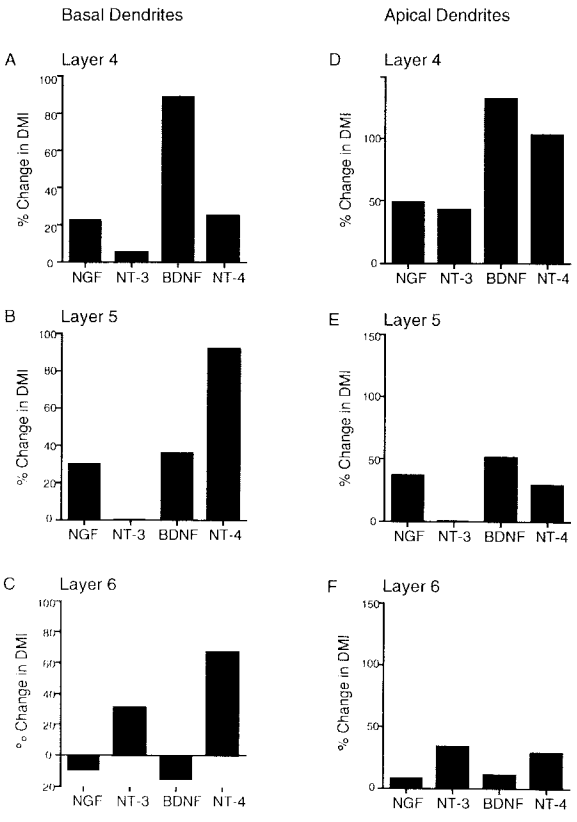

Figure 6. Changes in the DMI Caused by Neurotrophin Treatment

The dendrite modification index (DMI) for neurons in each cortical layer treated with the four neurotrophins was calculated as described in Experimental Procedures and presented in Table 2. For each layer, the change in DMI resulting from each neurotrophin treatment is shown. Basal dendrites (A–C) responded primarily to a single neurotrophin: neurons in layer 4 (A) were maximally enhanced by BDNF, whereas neurons in layer 5 (B) and layer 6 (C) were maximally enhanced by NT-4. Apical dendrites (D–F) showed a wider spectrum of response.

Biolistics Does Not Alter the Dendritic Development of Pyramidal Neurons

The approach used to visualize the dendritic trees of pyramidal cells, biolistics, involved exposing slices to accelerated DNA-coated gold particles to transfect individual neurons (see Experimental Procedures; also see Lo et al., 1994). Because we were concerned that the observed effects of neurotrophins might reflect neurotrophin-promoted recovery from this insult, rather than the growth-promoting effects of these factors, we did several control experiments. The most important of these was to employ a completely different method for visualizing neurons. To do this, we used intracellular injections of the fluorescent dye Lucifer yellow into individual neurons in fixed slices that had undergone treatment identical to the transfected slices except for the actual particle bombardment (see Experimental Procedures). These neurons were recon-

structed and analyzed using exactly the same approach used for transfected neurons. Comparison of Lucifer yellow–filled cells with identically treated, transfected neurons in layers 4 and 6 revealed no significant differences in any of the parameters used to construct the DMI: total dendritic length, number of dendrites and secondary branches, and number of protospines (Figure 7). In other experiments, we qualitatively compared immmunohistochemical staining against microtubule-associated protein 2 (MAP2), which stains cortical dendrites, in transfected and control slices and could detect no obvious differences (data not shown). We also observed no differences when we quantitatively compared cell number and cell density in 1 μm thick, Nissl-stained plastic sections taken from either transfected or control slices (data not shown). Thus, in this organotypic slice culture system, transfection of neurons using biolistics did not cause detectable neuronal damage.

Discussion

As summarized in Figure 8, dendritic growth of pyramidal neurons in specific cortical layers was potently modulated by the neurotrophin family of growth factors: NGF, BDNF, NT-3, and NT-4. As both the neurotrophins and their cognate receptors are present in the developing neocortex, this evidence implicates these factors in establishing final dendritic form in vivo.

Effects of Neurotrophins in Visual Cortex Are Similar to but More Complex than Effects in the PNS

In the PNS, systemic NGF increases dendritic complexity (Purves et al., 1988; Snider, 1988; Ruit and Snider, 1991). Similarly, neurotrophins enhanced dendritic growth and complexity of neurons in developing visual cortex. However, neurotrophin modulation of cortical dendritic growth was considerably more complex than in the periphery; neurons in each cortical layer responded only to certain neurotrophins, and even within a layer, neurons responded to each neurotrophin with a distinct constellation of dendritic changes (Figure 8). This specificity of neuronal responses to each neurotrophin may be crucial for the simultaneous development of the highly specific dendritic patterns characteristic of pyramidal neurons in each of the layers of cortex.

Neurotrophins could, in some cases (e.g., NGF and BDNF in layer 6), reduce dendritic elaboration, a phenomenon not previously reported for peripheral neurons. This indicates that, at least in neocortex, the same neurotrophin can regulate dendritic growth in both positive and negative directions. Such dual action of molecular cues in nervous system development has received attention from recent descriptions of netrin function (Colamarino and Tessier-Lavigne, 1995) and may represent a general developmental phenomenon.

Compartmental Specificity of Neurotrophin Responses

Pyramidal neurons have two separate dendritic compartments, each with a distinct arborization pattern. Basal dendrites are short, dense, and generally confined to the same cortical layer as the soma, while apical dendrites traverse several cortical layers before terminating with a "tuft" in layer 1 (Miller, 1988). This distinction between apical and basal dendrites is not only structural but also functional; apical dendrites probably receive distinctly different patterns of synaptic inputs than basal dendrites.

Neurotrophins differentially modulated basal and apical dendritic growth. In general, basal dendrites in each layer responded most strongly to a single neurotrophin, whereas apical dendrites responded to a different and wider array of neurotrophins (see Figure 6; Table 2). For neurons in layer 4, the effects of neurotrophins were roughly the same for both basal and apical dendrites. This is perhaps not surprising, as apical dendrites of layer 4 neurons are essentially confined to layer 4 at this age (layers 2/3 are just beginning to be formed at P14). In contrast, for layer 5 neurons, neurotrophin effects were different for basal and apical dendrites. This might be due to the fact that most of the apical dendritic tree of layer 5 neurons is actually located in layer 4, where BDNF is most effective; only a small section is located in layer 5, where NT-4 is the most effective ligand for basal dendrites.

This compartmental specificity of response within individual neurons suggests that apical and basal dendrites respond in distinct ways to the same extracellular signal. The branching of apical dendrites is frequently restricted to specific layers related to the functional roles of the parent neurons (Lund and Boothe, 1975). Here, application of neurotrophin to cortical slices sometimes caused extension of apical dendritic arbors beyond their normal projection range, particularly in BDNF-treated layer 4 neurons (see Figure 2). Thus, restricted expression of Trk receptors, coupled with focal production and secretion of neurotrophins, could be a mechanism for achieving the marked specificity of dendritic arborization seen during normal development.

BDNF and NT-4, Both TrkB Ligands, Have Distinct Effects on Cortical Dendrites

Because BDNF and NT-4 have reportedly similar specificity, potency, and affinity for TrkB receptors in neurons, their biological effects are generally assumed to be similar (Berkemeier et al., 1991; Ip et al., 1992, 1993; Klein et al., 1992). However, their effects on dendritic growth in every layer of visual cortex were distinct (Table 3). BDNF increased dendritic growth in layers 4 and 5 and decreased it in layer 6, whereas NT-4 enhanced dendritic growth in all layers (Table 3; see Figure 8). Even in layers 4 and 5, where both BDNF and NT-4 enhanced dendrites, these neurotrophins modulated different aspects of dendritic morphology (Table 3; see Figure 8).

Such marked differences imply that these two neurotrophins have distinct functions during cortical development. Indeed, recent gene deletion experiments in mice lacking BDNF and/or NT-4 have suggested separate roles for BDNF and NT-4 during development (Conover et al., 1995; Liu et al., 1995). Our observations extend this comparison by showing that BDNF and NT-4 not only affected different populations of neurons, as in these knockout studies, but also elicited unique cellular responses within

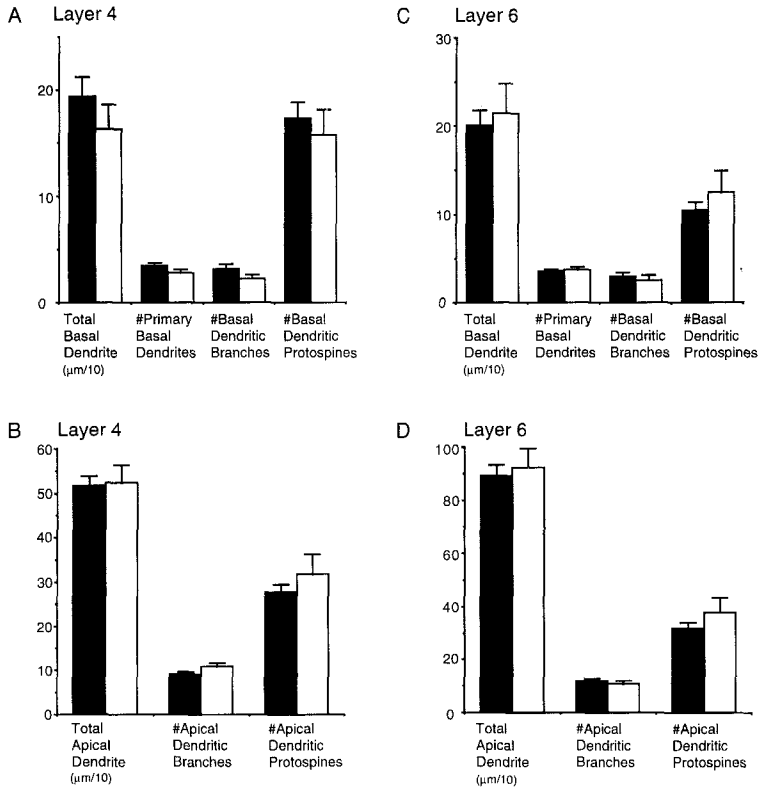

Figure 7. Dendritic Morphology of Neurons Visualized by Intracellular Injection Compared with Biolistic Transfection

Ferret (P14) visual cortex slices were either cultured for 36 hr, fixed, and injected intracellularly with Lucifer yellow, or transfected with *lacZ*, cultured for 36 hr, and visualized by immunocytochemistry. Comparison of four parameters of basal (A and C) and apical (B and D) dendritic growth of neurons in layer 4 (A and B) and layer 6 (C and D) showed no significant differences in fine aspects of neuronal morphology visualized by these methods. Closed bars represent means ± 1 standard error from intracellular fills (layer 4, n = 35; layer 6, n = 36). Open bars represent data from cells visualized by biolistic transfection (layer 4, n = 27; layer 6, n = 20).

the same neuronal population. These observations imply a difference either in binding of each ligand to specific isoforms of TrkB (Klein et al., 1992; Ip et al., 1993), in the signal transduction cascades activated subsequent to ligand binding of TrkB, and/or in interactions with as yet unknown neurotrophin receptors.

What Is the Molecular Basis for the Specificity of Dendritic Responses to Neurotrophins?

Because neurotrophins exert their effects through binding to Trk receptors, the dendritic responses to neurotrophins reported here are likely the result of neurotrophin interactions with cortical Trk receptors. Although we have no direct evidence that this is the case, the layer specificity of dendritic responses of neurons to neurotrophins was consistent in part with reported layer-specific expression of the Trk receptors. Both BDNF and NT-4 affected neurons in layer 5, where TrkB, their cognate receptor, is exclusively expressed in P14 ferrets (binding specificity: Berkemeier et al., 1991; Klein et al., 1991b; Ip et al., 1992, 1993; Klein et al., 1992; localization: Cabelli et al., 1993, Soc. Neurosci., abstract; Lindsay et al., 1994). NT-3 affected primarily dendrites of neurons located in layer 6 (see Figure 8; Table 1), where mRNA for its primary receptor, TrkC, has been localized by in situ hybridization in rat at a comparable developmental stage to the P14 ferret (binding specificity: Lamballe et al., 1991; Chao, 1992; localization: Ringstedt et al., 1993; Lindsay et al., 1994).

However, dendrites of neurons in several layers were enhanced by neurotrophins despite a lack of evidence for expression of the corresponding Trk receptor. For example, although TrkB is reportedly restricted to layer 5 at

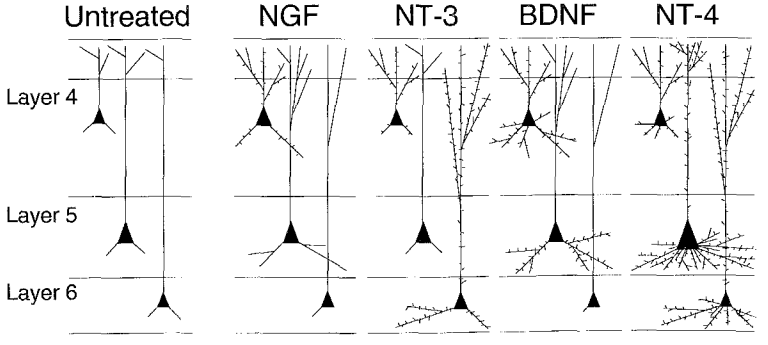

Figure 8. Summary of Neurotrophic Effects on Pyramidal Neurons in Developing Visual Cortex

The layer- and factor-specific effects of neurotrophins on neuronal dendrites are shown. Changes in total dendritic length, the number of primary dendrites, the number of dendritic branches, and the number of protospines are depicted schematically in these drawings. The most dramatic and consistent modifications of dendritic arborization were caused by the TrkB ligands BDNF and NT-4, although responses to these two factors were clearly distinct. NT-3 treatment enhanced dendrites of neurons mainly in layer 6, whereas NGF caused minimal dendritic changes in all cortical layers.

Table 3. Comparison of the Effects of BDNF versus NT-4 on Four Measures of Dendritic Growth for Pyramidal Neurons

	Layer 4		Layer 5		Layer 6	
	BDNF (52)	NT-4 (51)	BDNF (24)	NT-4 (28)	BDNF (42)	NT-4 (39)
Basal dendrites						
Dendritic length ($\mu m \times 10^{-1}$)	+++	0	++	+++	0	++
Number of primary dendrites	+++	+++	0	+++	−−	++
Number of branches	+++	0	++	+++		++
Number of protospines	+++	+++	+	+++	0	+++
Apical dendrites						
Dendritic length ($\mu m \times 10^{-1}$)	+++	+++	++	0	+	+
Number of branches	+++	+++	+++	+++	0	+++
Number of protospines	+++	+++	0	++	0	+++

The significance of change from untreated values was tabulated (0 = no significant change from untreated values; +/− = $p \leq .05$; ++/−− = $p \leq .01$; +++ = $p \leq .001$). The boxes delineate parameters in which there is a significant difference ($p \leq .05$) between values of growth for slices treated with BDNF compared with NT-4. Statistical significance was calculated by single-factor ANOVA.

this age (Cabelli et al., 1993, Soc. Neurosci., abstract), neurons in both layer 4 and layer 6 responded to BDNF and NT-4 (see Figure 8; Table 1). Also, NGF produced modest changes in dendritic complexity of neurons in layers 4, 5, and 6 (see Figure 8; Table 1), although neither TrkA nor the low-affinity NGF receptor, p75, has been detected in the cortical plate (Allendoerfer et al., 1990; Kaplan et al., 1991; Klein et al., 1991a; Chao, 1992; Merlio et al., 1992; Lindsay et al., 1994). There is, however, some precedent for functional responses of cortical neurons in kittens to NGF, as the effects of monocular deprivation in visual cortex were prevented by NGF infusion in vivo (Carmignoto et al., 1993).

The absence of correspondence between the layer specificity of effects of neurotrophins on dendritic growth and the reported localization of their receptors could arise in several ways. First and most likely, some of the localization results described above may not apply to the P14 ferret: most studies did not localize all isoforms of the Trk receptors at different developmental stages and most were performed on the rat (Allendoerfer et al., 1990; Merlio et al., 1992; Cabelli et al., 1993, Soc. Neurosci., abstract; Ringstedt et al., 1993). Alternatively, other as yet unidentified receptors may exist through which the neurotrophins exert their effects. Second, the effects observed here could be indirect effects through a subpopulation of responsive neurons in the cortical plate or subplate. Cells in the subplate of the ferret express p75, *trk*B, and *trk*C, and they project widely within the cortical plate at this age (Allendoerfer et al., 1990, 1994; Friauf et al., 1990). However, such indirect trophic interactions from these widespread subplate projections would not be expected to show the layer-specific effects of neurotrophins reported here. Finally, the neurotrophin effects described here could reflect cross-reactivity of exogenous neurotrophin to "nonpreferred" Trk receptors. However, the concentration of neurotrophin used in these experiments (200 ng/ml) is well below the concentrations at which cross-reactivity occurs (Ip et al., 1993). Moreover, the actual concentration experienced by neurons in slice is likely to be considerably lower.

Our results indicate that exogenous neurotrophic factors modulated the dendritic arborization of pyramidal neurons in visual cortex slices during a specific developmental window. Furthermore, dendritic responses to neurotrophins were not simple enhancements of dendritic growth but were specific modulations of particular patterns of dendritic arborization. As neurotrophins and their receptors are present in temporally and spatially restricted patterns in developing cortex (Allendoerfer et al., 1990; Castren et al., 1992; Chao, 1992; Merlio et al., 1992; Cabelli et al., 1993, Soc. Neurosci., abstract; Ringstedt et al., 1993), we propose that endogenous neurotrophins modulate specific patterns of cortical dendritic growth and branching in vivo. In support of this, recent studies have implicated neurotrophins in the development of cortical circuitry during ocular dominance column formation in developing ferret visual cortex (Cabelli et al., 1995). Future experiments addressing the cellular location of neurotrophin secretion and receptor expression should suggest more specific models by which such interactions occur. Finally, since both dendritic differentiation (Valverde, 1968; Harris and Woolsey, 1981; Katz and Constantine-Paton, 1988; Katz et al., 1989; Bailey and Kandel, 1993) and the expression of neurotrophins and their receptors (Castren et al., 1992; Lo, 1992; Patterson et al., 1992) are modulated by levels of neuronal activity, these results also implicate neurotrophins in activity-dependent growth and modification of dendritic arbors of pyramidal neurons during cortical development.

Experimental Procedures

Preparation of Visual Cortex Slice Cultures

Coronal slices (400 μm) of P14 ferret visual cortex were prepared under sterile conditions as previously described (Katz, 1987), except that the artificial cerebrospinal fluid was modified by substituting 10 mM HEPES buffer for $NaHCO_3$. Slices were incubated in 6-well sterile tissue culture plates on 0.4 μm Millicell inserts (Millipore) with 1 ml of media under each insert (50% Basal Medium Eagle, 25% Hanks' balanced salt solution, 25% horse serum [Hyclone], 330 mM dextrose, 10 mM HEPES, and 100 U/ml penicillin-streptomycin; all from GIBCO–BRL except horse serum). Culture plates were incubated in 5% CO_2 in air at 37°C.

Normal pigmented ferrets were obtained from Marshall Farms (North Rose, NY). For each treatment, approximately ten ferrets (P14) were used from five different litters. At P14, the age we used in these experiments, inputs from the lateral geniculate nucleus have arrived in layer 4, but the visual system is still highly immature: the eyes will not open for at least 2 more weeks, and photoreceptors in the retina are not yet born (Allendoerfer et al., 1994). In addition, neurons destined for layers 2/3 are still migrating through the cortical plate at this stage of development (McConnell, 1988; Jackson et al., 1989).

Particle-Mediated Gene Transfer

Transfection of cultured slices using a biolistics device (Bio-Rad) was

performed within 4 hr of slice preparation using a standard protocol (Lo et al., 1994). In brief, 0.6 mg of 1.6 μm gold particles were coated with 1 μg of plasmid DNA in 1.25 M $CaCl_2$, 100 mM spermidine-free base for each transfection. This preparation was scaled appropriately for each experiment to ensure that identically coated particles were used for transfection of slices in each condition. Particles were accelerated by an 1100 psi helium pressure transient into target slices that were placed ~9 cm away in a partial vacuum of 25–27 in of Hg. Slices were immediately returned to the incubator and maintained for 36 hr in the presence or absence of neurotrophins.

Intracellular Injections in Fixed Slices

Slices were prepared and cultured as described above but without particle-mediated gene transfer. After 36 hr in culture, the slices were immersion fixed for 1.5 hr with 2.5% paraformaldehyde, 4% sucrose and transferred to phosphate-buffered saline (PBS) for storage for up to 48 hr. Intracellular injection of Lucifer yellow (Molecular Probes) was performed using sharp electrodes as previously described (Katz, 1987). Slices were placed on the modified stage of a Zeiss microscope, and the electrode was manually directed into the tissue. Cells penetrated by the electrode were visualized using standard flourescent filters as they filled with the fluorescent dye. Lucifer yellow was iontophoresed with a 1–5 nA current generated from a Microiontophoresis Current Programmer (World Precision Instruments) using 200 ms pulses every 400 ms for 2–5 min provided by an A310 Accupulser (World Precision Instruments). Following intracellular injection, slices were postfixed for 1–3 hr, rinsed in PBS, dehydrated, defatted, and coverslipped for analysis.

Neurotrophins

Neurotrophin was added to a concentration of 200 ng/ml within 1 hr after transfection. Human recombinant neurotrophins rHu-met-BDNF, rHu-NT-3, and hNT-4 were generously provided by Regeneron Pharmaceuticals (Tarrytown, NY). Collaborative Biochemical was the source of 2.5S mouse NGF.

Histochemistry and Immunocytochemistry

The plasmid used in these experiments was EFβ-gal, in which expression of the *lacZ* gene is driven by the Xenopus EF1α promoter (Krieg et al., 1989). For histochemical visualization of β-galactosidase expression, slices were fixed in 0.5% glutaraldehyde and incubated in 1 mg/ml X-Gal (Molecular Probes), 17.5 mM potassium ferricyanide, 17.5 mM potassium ferrocyanide, and 1 mM $MgCl_2$ in PBS at 37°C for 18–24 hr.

For immunocytochemical visualization of β-galactosidase expression, slices were fixed in 2.5% paraformaldehyde with 4% sucrose and then saturated with 30% sucrose before freeze-thawing. Blocking serum contained 10% normal goat serum, 2% bovine serum albumin, and 0.25% Triton in 0.1 M phosphate buffer. Slices were incubated in a 1:500 dilution of an affinity-purified rabbit antibody to β-galactosidase (5′-3′) followed by a 1:100 dilution of rhodamine-conjugated goat anti-rabbit secondary antibody (Boehringer Mannheim).

Cell Selection, Reconstruction, and Analysis

Following histological processing, every neuron whose complete dendritic tree was filled and stained sufficiently to be reconstructed fully was selected for analysis. Sufficient filling was determined by unambiguous identification of the tips of all of the smallest dendritic processes. An additional requirement for selection was that the laminar position of the selected neuron be easily determined by double staining of the slice with 4′,6-diamidino-2-phenylindole (DAPI).

Epifluorescence photomicrography and camera lucida tracing were performed using standard filters for fluorescein and rhodamine on a Zeiss Axiophot microscope. Neurons were traced with a camera lucida using a 63× objective, and from these drawings, cell soma size and the parameters of dendritic growth were directly counted or measured with a digital plan measure. The four parameters that we chose to describe developing dendrites were the cumulative length of dendrite (μm) and the numbers of primary dendrites, higher order branches of each primary dendrite, and protospines (defined as spinelike protrusions greater than 5 μm in length on young neurons). Apical dendrites were defined as the single, thick dendrite that extended from the top of the neuronal soma to layer 1; basal dendrites were defined as those dendrites that extended radially from the soma.

Calculation of the DMI

To summarize the effects of each neurotrophin on dendritic development, we used the data on dendritic form shown in Table 1 to derive an index that accurately conveyed the magnitude and direction of neurotrophin-induced changes. The resulting DMI is simply a weighted sum of the ratios for each of the four parameters of dendritic growth in treated versus untreated cells. Each ratio was weighted to reflect our perception of the relative importance of a given parameter in determining dendritic morphology. For basal dendrites, we assigned the greatest weight to changes in the total length of dendrite, the next greatest weight to changes in the number of primary dendrites, then dendritic branching, and the least weight to changes in the numbers of protospines. The DMI for basal dendrites was thus calculated as:

$$\mathrm{DMI} = \sum_{i=1}^{4} C_i T_i / UT_i,$$

where T_i is the value of a given parameter i after neurotrophin treatment, UT_i is the value in untreated slices, and C_i is a normalized weighting constant whose magnitude depends on the parameter. For basal dendrites, we assigned: $C_1 = 0.4$ for total length of dendrite, $C_2 = 0.3$ for number of basal dendrites, $C_3 = 0.2$ for number of dendritic branches, and $C_4 = 0.1$ for number of protospines. The ratio T/UT was set to a value of 1 for cases in which treated values were not statistically distinct from untreated values ($p \leq .05$). Thus, for a neurotrophin treatment that had no statistically significant effect on basal dendrites, the DMI = (0.4 × 1) + (0.3 × 1) + (0.2 × 1) + (0.1 × 1) = 1. In the case of BDNF treatment of layer 4 basal dendrites (see Table 1): DMI = (0.4 × 340/180) + (0.3 × 5/3.2) + (0.2 × 6.4/2.8) + (0.1 × 34.3/16.7) = 1.89. This was an almost 90% increase in the DMI and reflected the marked changes in dendritic arborization caused by BDNF treatment (see Figure 2).

The DMI for apical dendrites was calculated similarly. However, since the number of primary dendrites is always 1 for apical dendrites (each cell has only one apical dendrite), only three parameters were assessed. The values for C_i were: $C_1 = 0.5$ for total dendrite, $C_2 = 0.4$ for number of dendritic branches, and $C_3 = 0.1$ for number of protospines.

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