

Opposing roles for neurotrophin-3 in targeting and collateral formation of distinct sets of developing cortical neurons

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SUMMARY

Neurotrophin-3 and its receptor TrkC are expressed during the development of the mammalian cerebral cortex. To examine whether neurotrophin-3 might play a role in the elaboration of layer-specific cortical circuits, slices of layer 6 and layers 2/3 neurons were cultured in the presence of exogenously applied neurotrophin-3. Results indicate that neurotrophin-3 promotes axonal branching of layer 6 axons, which target neurotrophin-3-expressing layers in vivo, and that it inhibits branching of layers 2/3 axons, which avoid neurotrophin-3-expressing layers. Such opposing effects of neurotrophin-3 on axonal branching were also observed with embryonic cortical neurons, indicating that the response to neurotrophin-3 is specified at early developmental stages, prior to cell migration. In

addition to its effects on fiber branching, axonal guidance assays also indicate that neurotrophin-3 is an attractive signal for layer 6 axons and a repellent guidance cue for layers 2/3 axons. Experiments with specific antibodies to neutralize neurotrophin-3 in cortical membranes revealed that endogenous levels of neurotrophin-3 are sufficient to regulate branching and targeting of cortical axons. These opposing effects of neurotrophin-3 on specific populations of axons demonstrate that it could serve as one of the signals for the elaboration of local cortical circuits.

Key words: Cortical development, Wiring molecule, Neurotrophin, Cortical circuit, Axonal targeting, Axonal branching, Rat

INTRODUCTION

Over the past few years, new functions have been attributed to the neurotrophins, a family of secreted proteins that have been implicated in regulating neuronal survival, cell proliferation and differentiation (for review see Korsching, 1993; Snider, 1994). From the initial finding that NGF enhanced axonal growth of sympathetic neurons and attracted regeneration axons towards a source of NGF (Menesini-Chen et al., 1978; Gundersen and Barret, 1978; Letourneau, 1978), a number of studies provided evidence for a role of neurotrophins in the regulation of neuritic morphology in several regions of the developing nervous system (Cohen-Cory and Fraser, 1995; Inoue and Sanes, 1997; McAllister et al., 1995). Analysis of NT-3 mutant mice showed that sympathetic neurons failed to innervate two target structures, therefore suggesting a role for NT-3 in axonal pathfinding and/or target recognition (Elshamy et al., 1996). In sympathetic neuronal cultures, focal application of NT-3 induced a turning of the growth cone towards the source, providing a direct evidence for chemoattractive effect of NT-3 (Paves and Saarma, 1997). In addition, NT-3 was found to enhance axonal sprouting of corticospinal neurons (Schnell et al., 1994), and to exert a dual effect on the dendritic branching of layer 6 and layer 4 neurons in the developing visual cortex (McAllister et al., 1997).

In the developing cerebral cortex, the expression pattern of NT-3 and its high-affinity receptor TrkC is consistent with a possible role of NT-3 in the formation of cortical connections. In rodents, a number of previous studies showed that both NT-3 protein and its mRNA are detected in the cerebral cortex at embryonic and postnatal stages (Maisonpierre et al., 1990; Ernfors et al., 1992; Fukumitsu et al., 1998; Friedman et al., 1998). Expression is particularly high in the cingulate cortex (Friedman et al., 1991, 1998; Söderström and Ebendal, 1995) but it is also detected in several neocortical regions (Lauterborn et al., 1994; Schoups et al., 1995; Söderström and Ebendal, 1995). During development, NT-3 expression is temporally regulated. It is found from early embryonic stages in the cerebral cortex and postnatally, the expression decreases with maturation (Friedman et al., 1991, 1998; Ernfors et al., 1992; Söderström and Ebendal, 1995; Fukumitsu et al., 1998). Moreover, in higher mammals, it has been shown that NT-3 mRNA exhibits a laminar distribution, being found in layer 6 and layer 4 of the developing visual cortex (Lein et al., 1995, 1997; Chaudhuri, 1997). In the rodent cerebral cortex, both TrkC receptor and its mRNA are detected in the cortical plate, subplate and ventricular zone from early prenatal stages (Ernfors et al., 1992; Fukumitsu et al., 1998). During the first postnatal days, TrkC mRNA is predominantly expressed in layer 6 and, during the first two postnatal weeks, at the time

when local connections are being formed, the expression is mainly found in layer 6 and layers 2/3 (Ringstedt et al., 1993). This layer-specific expression is maintained at adult stages (Merlio et al., 1992; Ringstedt et al., 1993; Valenzuela et al., 1993; Lamballe et al., 1994; Fukumitsu et al., 1998). In addition, studies of neurotrophin receptor responsiveness indicate that NT-3 receptors are functional during prenatal and postnatal cortical development (Knüsel et al., 1994).

In the mammalian cerebral cortex, neurons located in different cortical layers send axons to distinct cortical or subcortical targets, and their axon collaterals give rise to local stereotyped connections between the cortical layers (Lund and Boothe, 1975; Gilbert and Wiesel, 1979; Martin and Whitteridge, 1984). For example, pyramidal neurons in deep and superficial layers exhibit complementary patterns of interlaminar connections and, during development, they select the appropriate cortical layers from the onset of collateral outgrowth (Lund et al., 1977; Meyer and Ferres-Tores, 1984; Katz, 1991; Callaway and Lieber, 1996). In previous work, we found that subsets of cortical neurons respond to axonal guidance and branching signals expressed in distinct cortical layers, indicating that positional cues regulate the laminar specificity of local cortical circuits (Castellani and Bolz, 1997a; Castellani et al., 1998). The functional properties of NT-3 and its expression pattern in the developing cortex suggest that it might act as one of the signals that specify intrinsic cortical projections. To address this issue, in the present study, we analyzed the influence of exogenous NT-3 on layer 6 and layers 2/3 neurons and found that it exerts opposing effects on axonal elongation, branching and trajectory of these two sets of axons. To determine whether endogenous NT-3 was capable of inducing an axonal response of cortical neurons, we used specific antibodies to block NT-3 in cortical membranes. Results demonstrated that physiological levels of NT-3 are sufficient to regulate the growth and branch formation of cortical neurons.

MATERIALS AND METHODS

Preparation of cortical explants

Cortices from embryonic (E)15 and E16 rat embryos (E1, first day of gestation) were dissected under a microscope with oblique illumination and cut into 200×200×200 µm cubes with a McIlwain tissue chopper. At these developmental stages, the cortical plate is composed of subplate neurons, the earliest cells produced, and layer 6 neurons. In the ventricular zone, precursor cells are producing neurons predominantly destined for layer 6 (Miller, 1988; Bayer and Altman, 1991). During corticogenesis, neurons destined for distinct layers are produced sequentially and at late developmental stage, deep layer cells have migrated out of the ventricular zone. At that time, the ventricular zone contains postmitotic and mitotic neurons that will establish layers 2/3. We therefore dissected cortices from E19 embryos and isolated the ventricular zone to prepare explants containing neurons destined for layers 2/3.

Preparation of postnatal slices from isolated cortical layers

Cortical layers were isolated from P7 rat as described in Castellani and Bolz (1997a). Briefly, blocks of cortex were dissected in Gey's balanced salt solution supplemented with 6.5 mg/ml glucose and cut with a tissue chopper in 300 µm thick slices. Under a dissecting microscope with oblique illumination, the slices were cut along the

laminar borders between individual layers. Layer 6 and layer 5 differed in the transparency of the tissues. Layer 4 formed a bright tissue allowing to delineate the boundaries with adjacent cortical layers. A first cut was made along the border between layer 4 and layer 5, which is clearly visible, layer 5 being more transparent than layer 4. The boundary between layer 4 and layer 3 appeared more diffuse and we therefore made a second cut in the region located just above this zone, which corresponds to the deeper part of layer 3, to completely remove layer 4 from the rest of the tissue. The landmarks used in the dissection were confirmed by Nissl staining of entire and dissected cortical slices.

Preparation of membrane substrata

Membranes were prepared as described in Götz et al. (1992). P5 postnatal cortices were dissected and collected in an homogenization buffer. Cortical layers were dissected as described above and collected separately for the membrane purification. The optical densities (OD) of the membrane suspensions were measured with a spectrophotometer at 220 nm. OD was adjusted to 0.1 after 15-fold dilution in 2% SDS. In some experiments, NT-3 (Promega, 200 ng/100 µl of membrane suspension) or polyclonal antibodies directed against NT-3 (Promega, 1 µg/ml, in western blot experiments performed by the manufacturer, the antibody did not cross-react with the others neurotrophins) were incubated in the membrane preparations for 2-4 hours at 4°C. The suspension was then centrifuged at 14,000 revs/minute in an Eppendorf biofuge to remove unbound NT-3 and NT-3 antibodies.

Culture procedure

To prepare uniform membrane carpets, sterile glass coverslips were first incubated as a sandwich with 100 µl of Geys's balanced salt solution (GBSS) containing 1.5 µg laminin and 0.5 µg poly-L-lysine for 2 hours at 37°C. These coated coverslips were then washed with PBS and incubated again as a sandwich with 100 µl membrane suspension for 3 hours at 37°C. After separation, the coverslips were placed in a Petriperm dish with 750 µl culture medium. In some experiments, NT-3 was applied to the culture medium (100 ng/ml). Substrata composed of alternating membrane stripes were prepared as described in Walter et al. (1987). Alternating narrow stripes of NT-3 were prepared as described in Vielmetter et al. (1990). Briefly, glass coverslips were coated with 1.5 µg laminin and 0.5 µg poly-L-lysine, washed with PBS, dried and placed top to the channel matrix. 50 µl of NT-3 (10 ng/coverslip) was injected with a syringe into the channels. After incubation for 2 hours at 37°C, the channels were washed twice with PBS. Using phase contrast, alternating NT-3 and control lanes were identified by their differences in reflectance and thickness (Vielmetter et al., 1990). In some experiments, NT-3 (10 ng/coverslip) mixed with NT-3 antibodies (1: 50) was preincubated for 2-4 hours at 4°C and then injected into the channels. The coverslips were placed in a petriperm dish and covered with 750 µl of medium. Tissue was then explanted on the coverslips and culture medium was adjusted to 2 ml. Cultures were kept for 2 days at 37°C under 5% CO₂ in air atmosphere and then fixed with 4% PFA and 3% sucrose.

Slice cultures were prepared as described in Castellani and Bolz (1997b). The slices were stored in Gey's balanced salt solution for 45 minutes at 4°C, placed on a glass coverslip and embedded in 20 µl of chicken plasma coagulated with 20 µl of thrombin (0.2 mg/ml, 20 NIHU/ml Sigma). The cultures were incubated for 2 days at 37°C under continuous rotation, with NT-3 (200 ng/ml) applied in the culture medium from the onset of the culture period. To visualize axonal outgrowth, small crystals of lipophilic tracer DiI were inserted at different places in the slice. The cultures were then fixed with 4% PFA and 3% sucrose and kept for several days to allow diffusion of DiI.

Analysis of axonal growth, branching and targeting

To confirm the neuronal origin of the processes extending from the

explants, immunostaining was performed with the neuron-specific marker antibody SMI312 (Sternberger and Meyer Inc), which is directed against highly phosphorylated axonal epitopes of neurofilaments (Ulfig et al., 1998). Analysis of axonal growth and branching on uniform substrata was done as described before (Castellani et al., 1998). Axons extending from the explants were counted using a $\times 20$ phase-contrast objective (Zeiss Plan-Neofluar, N. A. 0.50). All explants were analyzed. To quantify axonal length, 10 explants per coverslip were randomly selected and the six longest fibers were measured. Statistic comparison was done using the Student's *t* test. To quantify axonal branching, explants were examined at higher magnification using a $\times 40$ phase-contrast objective (Zeiss Plan-Neofluar, N. A. 0.75) in combination with additional magnification lenses ($\times 1.6$ Optovar). Distal 100-200 μm segments of individual axons was analyzed. About 5 axons per explant were selected; crossing fibers and axon fascicles were not analyzed. The number of side branches was counted and the length of each axonal segment was measured. Axonal branching was then defined as the ratio between the total number of axonal branches and the total segment length. Axonal branching under control conditions was normalized to 100%. Statistic analysis was done with a permutation test. In the stripe assays, axons growing in each set of lanes were analyzed as described previously (Castellani and Bolz, 1997a). Data are presented in percentage. DiI-labeled axons in slice cultures were examined with a fluorescence microscope, objective $\times 40$, and collaterals were counted along the axons. Statistic analyses was done using Student's *t*-test.

RESULTS

Effect of NT-3 on axonal branching and elongation of cortical axons

Cortical slices prepared from E15 and E16 embryos, a stage at which the cortex contains postmitotic neurons predominately destined for layer 6, and from E19 ventricular zone when neurons destined for layers 2/3 are being produced (Miller, 1988; Bayer and Altman, 1991), were cultured in a plasma clot with NT-3 added to the culture medium. Analysis of the number of axon collaterals revealed that NT-3 increased the branch formation of layer 6 axons by 105%, compared to control conditions (total of 91 axons examined from two sets of experiments, $P < 0.0001$), whereas it decreased the branching of layers 2/3 axons by 39% (total of 137 axons examined in three separate experiments, $P < 0.001$, Fig. 1A,B). Previous work have shown that the elaboration of local axonal circuits occurs late during development, when the laminar architecture of the cortex is established (Katz, 1991; Callaway and Lieber, 1996). Therefore, to examine whether NT-3 could induce a branching response at a time when cortical neurons elaborate local connections, we isolated slices of layer 6 and layers 2/3 from P7 rat cortex and analyzed the formation of axon collaterals. Addition of NT-3 to the culture medium resulted in pronounced changes in axonal branching. In presence of NT-3, the number of axon collaterals of layer 6 neurons increased by 45% (214 axons examined in three different experiments,

$P < 0.01$), whereas axonal branching of layers 2/3 neurons was reduced by 47% (158 axons examined in three different experiments, $P < 0.0001$; Figs 1C-H, 2). These data indicate that

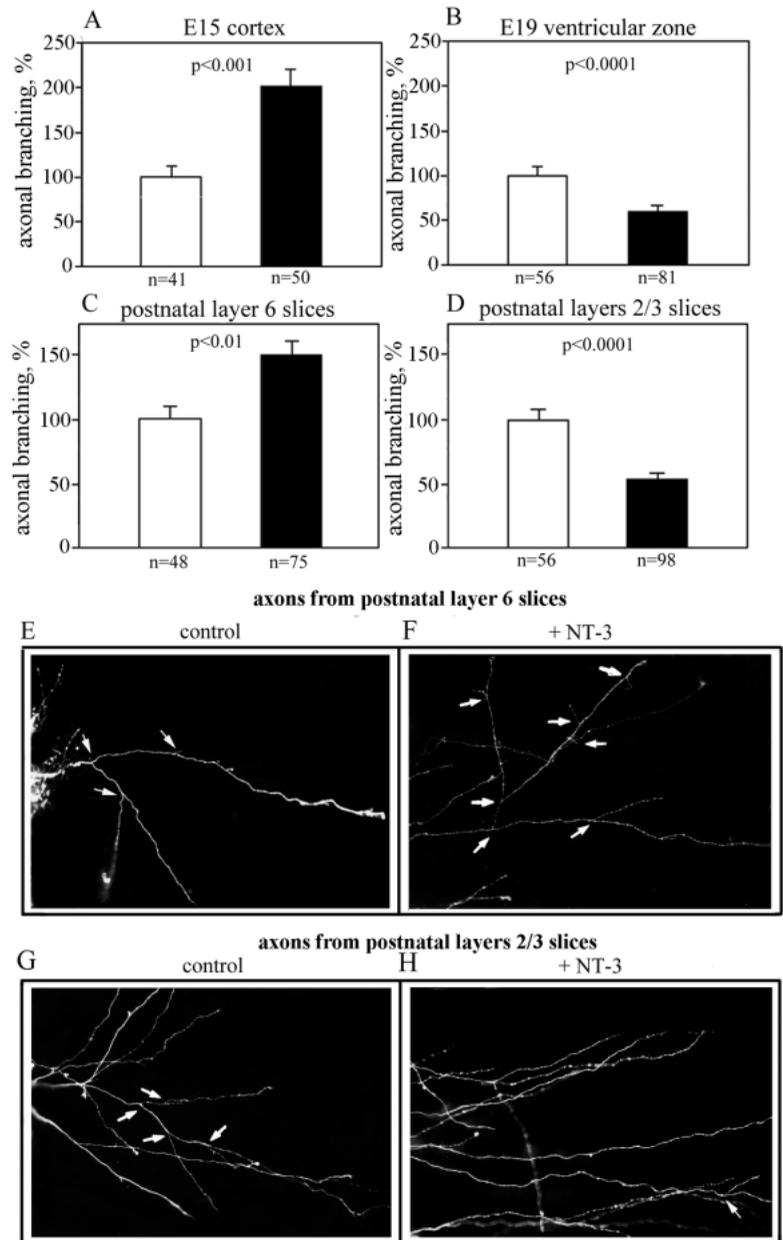


Fig. 1. (A-D) Quantitative analysis of collateral formation in embryonic (A,B) and postnatal slice cultures (C,D). Layer 6 and layers 2/3 slices were dissected from cortical slices prepared at P7, embryonic explants were prepared from whole E15 cortex, at a time when layer 6 neurons are generated, and from E19 isolated ventricular zone, when neurons destined for layers 2/3 are being produced in this region. Black bars represent the number of axon collaterals formed in presence of NT-3, white bars are control conditions. Overall, postnatal neurons elaborated more axon collaterals than embryonic neurons, however the effect of NT-3 on axonal branching was similar in both cases: for layer 6 neurons, application of NT-3 led to an increase in the number of axon collaterals, whereas the converse was true for layers 2/3 neurons. Errors bars represent SEM, n corresponds to the number of axons examined. (E-H) Fluorescence photomicrographs of DiI-labeled axons extending from postnatal cortical slices in the plasma clot. Dual effect of NT-3 on axonal branching of these two populations of cortical neurons, some axonal branch points are indicated by arrows. Scale bar, 20 μm .

NT-3 elicits similar cell-type-specific responses from embryonic neurons, which have not yet migrated into their final cortical positions, and from young postnatal neurons, which have reached their distinct layer in the cortex. However, as illustrated in Fig. 2, we also observed differences in the morphology of axon collaterals between embryonic and postnatal neurons. Axons from postnatal neurons extended long collaterals that were often tipped with a growth cone, and there were only few side branches that resembled filopodia. In contrast, most collaterals formed along axons from embryonic neurons were filopodia-like branches (Fig. 2). Overall, axonal branching was higher for postnatal neurons compared to embryonic neurons. Under control conditions, the number of axon collaterals was 3.4 and 2.2 times larger for layer 6 neurons and layers 2/3 neurons, respectively.

To examine the effects of NT-3 on axonal growth and elongation, cortical explants were cultured on a two-dimensional laminin-poly-L-lysine substratum, with NT-3 applied in the culture medium and in control conditions. This substratum did not provide appropriate growth conditions for postnatal tissue. The experiments described below were therefore performed only with embryonic tissue. In the rest of this paper and in the figures, we refer to neurons destined for layer 6 as neurons from 'E16 cortex' and to neurons destined for layers 2/3 as neurons from 'E19 ventricular zone (E19 VZ)'.

Analysis of axonal length revealed that axons of E16 neurons grew longer and axons of E19 VZ neurons grew shorter in presence of NT-3. As depicted in Fig. 3A, for E16 neurons, 50% of axons reached a length of 391 μm in control conditions and 479 μm in presence of NT-3 (420 axons measured in three separate experiments, Fig. 3A). In contrast for E19 VZ neurons, the length reached by 50% of axons decreased from 203 μm in control conditions to 162 μm when NT-3 was applied to the culture medium (120 axons from two distinct experiments, Fig. 3B). The effects of NT-3 on axonal branching were qualitatively comparable to those described above, when cortical slices were cultured in a 3-dimensional plasma clot. Application of the neurotrophin led to a 83.4% increase in axonal branching of neurons from E16 cortex (total of 155 axons examined, $P < 0.01$). In contrast, NT-3 treatment led to a 25% decrease of axonal branching, for E19 VZ neurons (total of 58 axons examined, $P < 0.05$; Fig. 3C,D). However, the number of axons extending from the explants was not influenced by NT-3. For E16 neurons, the average fiber outgrowth in absence and presence of NT-3 was 13.9 ± 1.9 and 16.1 ± 1.5 , respectively (83 explants examined, $P > 0.05$). Similarly for E19 VZ neurons, the number of axons extending from the explants in presence and absence of NT-3 was 6.0 ± 0.9 and 7.5 ± 1.0 , respectively (89 explants examined, $P > 0.05$).

Because axonal outgrowth was relatively low on laminin-poly-L-lysine substrata, in additional experiments, explants were cultured on postnatal cortical membranes, which are known to promote the outgrowth of cortical fibers (Götz et al., 1992). In this more complex molecular environment, the effects of NT-3 on axonal branching were similar to those observed on laminin-poly-L-lysine-coated coverslips. Compared to control conditions, axonal branching of E16 neurons increased by 49% when NT-3 was applied to the cultures (73 axons examined from two separate experiments, $P < 0.0001$; Table 1). For E19 VZ neurons, NT-3 reduced the

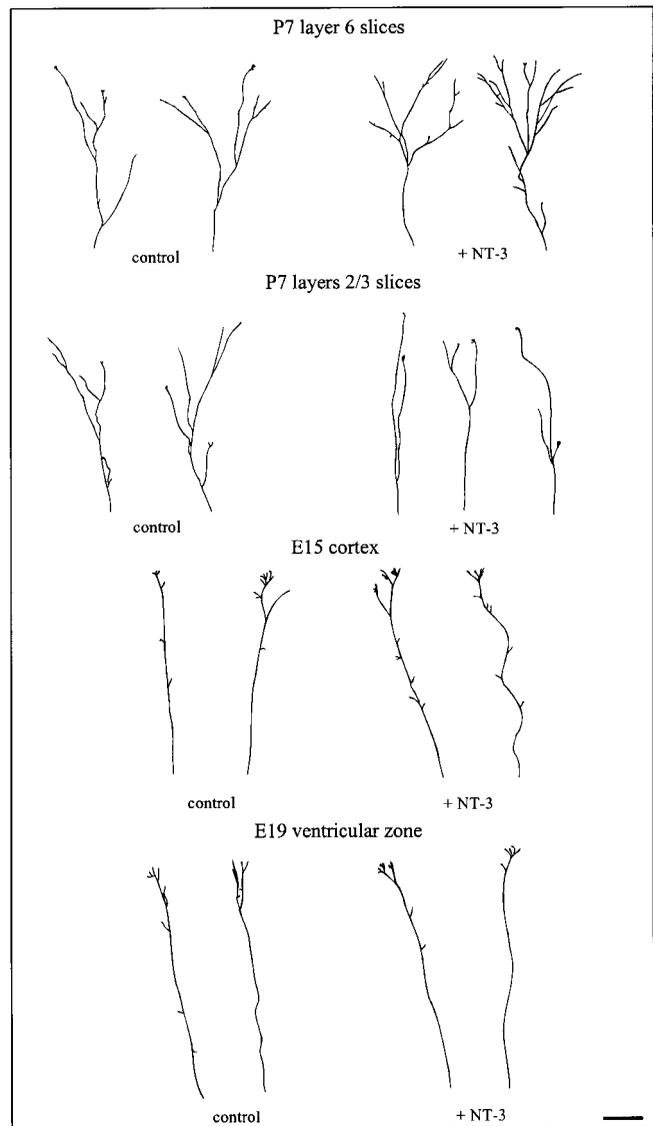


Fig. 2. Drawings of representative axons extending from postnatal and embryonic cortical explants in a plasma clot. From top to bottom: axons from postnatal layer 6 neurons (P7 layer 6 slices), from postnatal layers 2/3 neurons (P7 layers 2/3 slices), from embryonic neurons destined for layer 6 (E15 cortex) and layers 2/3 (E19 ventricular zone). In each row, left: axons grown in the absence of NT-3 (control), right: axons grown in the presence of NT-3 (+ NT-3). NT-3 increases axonal branching of both postnatal neurons in layer 6 and embryonic neurons destined for layer 6. In contrast, axon collateral formation of postnatal neurons in layers 2/3 and embryonic neurons destined for layers 2/3 was reduced by the application of NT-3. See text for further details. Scale bars, 100 μm .

formation of axon collaterals by 30% (62 axons examined from two separate experiments, $P < 0.001$; Table 1). As was the case on laminin-poly-L-lysine substrata, the number of fibers extending from cortical explants was not influenced by the presence of exogenously applied NT-3 (Table 1). However, in contrast to the results obtained with laminin-poly-L-lysine substrata, NT-3 had no effect on the elongation of axon growing on cortical membrane (Table 1). Thus, NT-3 might

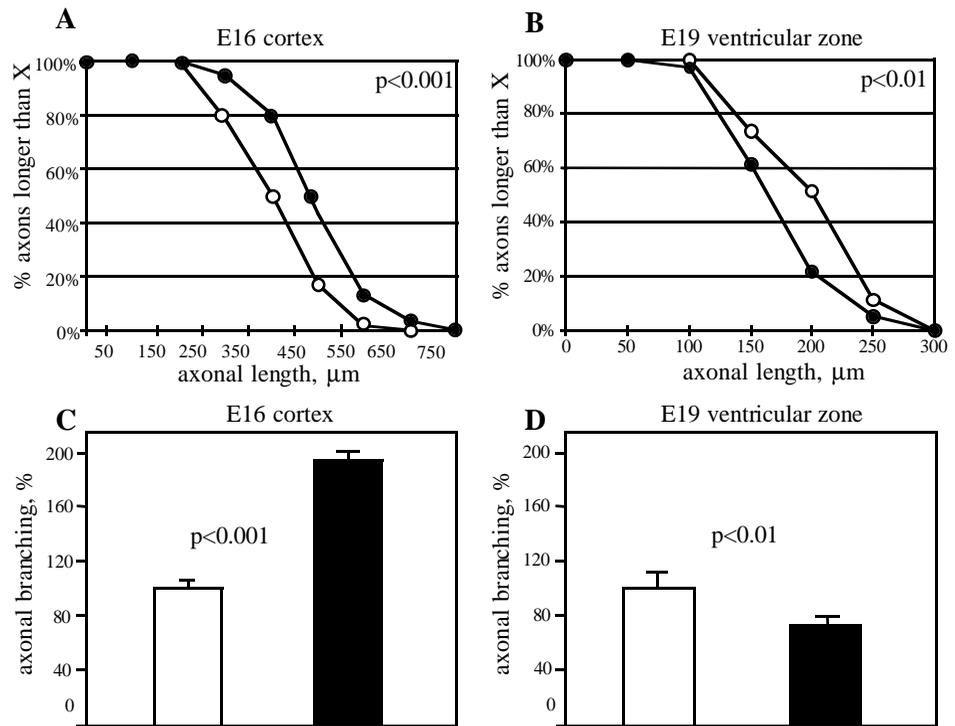


Fig. 3. Quantitative analysis of axonal growth and branch formation of neurons from E16 cortex and E19 VZ cultured on laminin-poly-L-lysine substrata. Black bars, NT-3 was applied to the culture medium, white bars correspond to the control conditions. (A,B) Axonal length, the plots represent the distribution of axonal length, Y-axis shows the proportion of axons that reached the length indicated on the X-axis. In presence of NT-3, axons from E16 neurons grew longer, whereas axons from E19 VZ neurons grew shorter than in control conditions. (C,D) In these cultures on two-dimensional substrata, exogenous NT-3 regulates axonal branching, as observed for cortical slices growing in a plasma clot.

promote the elongation of cortical axons only under suboptimal growth conditions, like on a laminin-poly-L-lysine substratum.

Substratum-bound NT-3 influences axonal trajectories of cortical axons

To examine a possible role of NT-3 in the targeting of cortical axons, we examined fiber outgrowth of explants cultured on narrow alternating stripes of NT-3. In this *in vitro* assay, axons were offered a choice between substrata consisting of lanes with laminin-poly-L-lysine alone and lanes composed of laminin-poly-L-lysine and NT-3. For E16 neurons, axons exhibited a clear preference for the lanes containing NT-3: 68.9% axons first emerged and then elongated in the lanes with NT-3 (109 explants examined in four separate experiments, *P*<0.0001, Fig. 4A-C). Under these conditions, the growth-promoting effect of NT-3 for this population of cortical axons was clear since the average axonal length increased from 174±12.3 µm in the control lanes to 291.4±14.8 µm in the lanes containing NT-3 (79 axons examined, *P*<0.0001). In contrast, 70.3% of axons from E19 VZ neurons avoided the NT-3 lanes and grew preferentially in the laminin-poly-L-lysine lanes (105 explants examined in four separate experiments, *P*<0.0001; Fig. 4A-C). To confirm that these effects were due to NT-3, cortical explants were cultured on alternating lanes of NT-3 preincubated with antibodies directed against NT-3 and control lanes. Under these conditions, cortical axons were no longer influenced by the stripe borders: 55% of layer 6 axons and 48% of layers 2/3 axons grew on the lanes containing NT-3 blocked with antibodies (60 explants examined in two sets of experiments, not significant).

Because NT-3 clearly influenced the axonal length when cortical explants were cultured on a laminin-poly-L-lysine substratum, the restricted growth observed in this stripe assay could reflect the ability of NT-3 to promote or inhibit the

elongation of cortical fibers, rather than regulating the trajectories of these axons (see previous section). Therefore we also examined whether axons growing in a more complex environment, providing better growth conditions, still respond to stripe borders of substratum-bound NT-3. Cortical explants were first cultured on a uniform substratum of cortical membranes preincubated with NT-3. As depicted in Table 2, we found that, under these conditions, neither the number of axons extending from the explants nor the fiber length were influenced by the presence of NT-3 in the substratum (two separate experiments, 80 explants examined for E16 neurons and 69 for E19 VZ neurons). However, when growing on alternating stripes of control membranes and NT-3 treated membranes, axons from E16 cortex clearly preferred to grow in the membrane lanes with NT-3 (137 explants examined in

Table 1. Quantitative analysis of axonal growth and branching of cortical neurons cultured on cortical membranes, with NT-3 applied in the medium

| | E16 cortex | E19 ventricular zone |
|-------------------------------|-----------------------|----------------------|
| Axonal branching | | |
| Control | 100±8 | 100±9 |
| +NT-3 | 149±9, <i>P</i> <0.01 | 70±6, <i>P</i> <0.05 |
| Fibre outgrowth | | |
| Control | 36.5±2.3 | 33.8±2.0 |
| +NT-3 | 41.6±2.5, n.s. | 36±3.1, n.s. |
| Axonal elongation, L50 | | |
| Control | 549 µm | 228 µm |
| +NT-3 | 561 µm, n.s. | 214.5 µm, n.s. |

NT-3 increased axonal branching for E16 neurons and decreased branching for E19 VZ neurons. For both populations, the length reached by 50% of the axons (L50), as well as the average number of axons per explant (fiber outgrowth), were not different in presence and absence of NT-3. The numbers are given ± s.e.m.; n.s., not significant.

four separate experiments, $P < 0.0001$; Table 2). On the contrary, E19 VZ axons exhibited the opposite behavior (83 explants examined in three separate experiments, $P < 0.0001$; Table 2). Taken together, these control experiments indicate that substratum-bound NT-3 can influence axonal trajectories and define growth-permissive and non-permissive territories for subsets cortical fibers.

Endogenous NT-3 regulates the targeting and branch formation of cortical axons

The results described so far indicate that cortical axons respond to exogenously applied NT-3. Does endogenous NT-3 exert similar effects? Is there sufficient amount of NT-3 present in the developing cortex to induce a response of cortical axons, and is the action of NT-3 restricted to individual cortical layers? Since NT-3 is a secreted molecule, a local action of this factor would require that it binds to cell surface or extracellular matrix components of the cortex. If endogenous NT-3 is present in cortical membrane preparations, and if it has an effect on axonal growth, then neutralizing NT-3 with specific antibodies should elicit effects opposite to those observed with exogenously applied NT-3. Thus, to examine whether physiological levels of NT-3 elicit a response of cortical neurons, we first cultured E16 and E19 VZ explants on postnatal cortical membranes preincubated with NT-3 antibodies. On uniform carpets of treated membranes, axonal branching of layer 6 neurons was reduced by 45% (173 axons examined in three separate experiments, $P < 0.001$) whereas the branch formation was increased by 43% for layers 2/3 neurons (68 axons examined in two separate experiments, $P < 0.001$), compared to the control substrata (Fig. 5A,B). Analysis of axonal length indicated that the antibody treatment had no effect on axonal length (data not shown).

To examine whether endogenous NT-3 influences the targeting of cortical axons, explants were cultured on alternating lanes of cortical membranes treated with NT-3 antibodies and native membranes. In this stripe assay, 65% of axons from E16 cortical explants preferred to grow in the lanes with native membranes rather than in lanes with membranes preincubated with neutralizing NT-3 antibodies (33 explants examined in two separate experiments, $P < 0.001$). The converse was true for E19 VZ neurons: the majority of axons (58.2%) grew in the treated membrane lanes (23 explants examined in two separate experiments, $P < 0.005$; Figs 5C,D, 6). Thus, both axonal branching and targeting can be regulated by endogenous levels of NT-3 present in postnatal cortical membranes. The effects on axonal targeting with membranes incubated with antibodies are less pronounced than the effects in the experiments with substratum-bound NT-3. Possible explanations for this are that the antibodies did not completely block NT-3 or that the NT-3 concentration in membranes prepared from all cortical layers is relatively low.

We also cultured cortical explants on membranes prepared from different cortical layers, preincubated with antibodies directed against NT-3. As indicated in Fig. 7A, axonal branching of E16 neurons decreased by

64.7% on treated membranes from layers 1-4 and by 44.8% on treated membranes from layer 6. In contrast, there was no significant difference in axonal branching on treated and untreated layer 5 membranes (399 axons examined in three separate experiments). For E19 VZ neurons, antibody preincubation increased axonal branching by 62.8% and 79.2% on membranes from layers 1-4 and layer 6, respectively, but there was again no effect with layer 5 membranes (167 axons examined in two separate experiments, Fig. 7B). These findings indicate that endogenous NT-3 functions as a regulator for axonal branching in cortical membranes prepared from layer 6 and layers 1-4. In contrast, layer 5 membranes either do not contain NT-3 or this factor is not efficient to induce axonal branching.

There are several possible explanations for the absence of

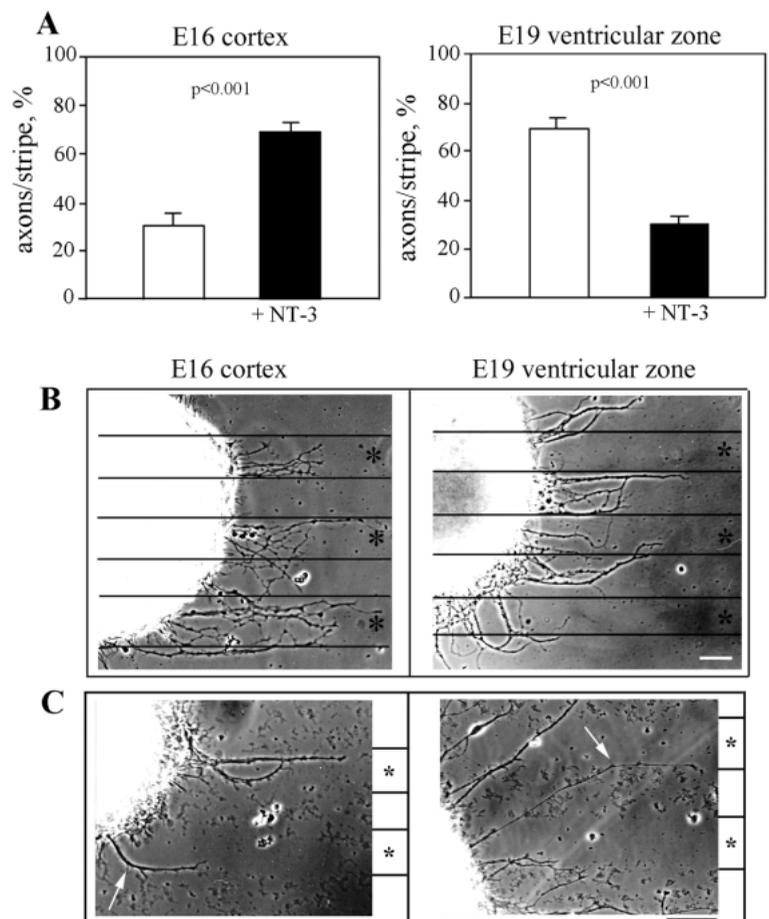


Fig. 4. (A) Quantitative analysis of axonal guidance in stripe assays. Cortical explants were cultured on alternating lanes of laminin-poly-L-lysine with and without NT-3. Histograms represent the percentage of axons which grew in each type of lanes, black bars correspond to the lanes with NT-3, white bars to the control lanes. E16 axons grew predominantly in the NT-3 lanes whereas axons from E19 VZ neurons preferred to elongate in the control lanes, in more than 70% of the cases. Errors bars represent SEM. (B) Phase-contrast photomicrographs from the stripe assays, illustrating the preferential growth of E16 axons for NT-3 lanes and of E19 VZ axons for the control lanes. (C) High magnification from layer 6 and layers 2/3 explants. As indicated by the arrows, axons reoriented their growth at a stripe border and maintained their trajectory within either NT-3 or control lanes. NT-3 lanes are identified by *. Scale bar, 50 μ m.

Table 2. Quantitative analysis of axonal growth of E16 and E19 VZ neurons cultured on cortical membranes previously incubated with NT-3 offered as a uniform substratum and in stripe assays

| | E16 cortex | E19 Ventricular zone |
|------------------------|------------------|----------------------|
| Fibre outgrowth | | |
| Control | 40.1±2.6 | 38±2.7 |
| +NT-3 | 42.05±2.8, n.s. | 36.4±3.2, n.s. |
| Axonal elongation, L50 | | |
| Control | 660 µm | 225 µm |
| +NT-3 | 680 µm, n.s. | 212 µm, n.s. |
| Axon/stripe | | |
| Control mbes | 31.8%±2.31 | 65.3%±4.00 |
| mbes+NT-3 | 68.2%±2.76 | 34.7%±4.57 |
| | <i>P</i> <0.0001 | <i>P</i> <0.0001 |

As indicated for both populations, no significant effect of NT-3 was noticed on fiber outgrowth and length. In contrast in the choice assay, E16 axons preferred to grow in the lanes with NT-3 bound to the membranes whereas E19 VZ axons preferred the control membrane lanes.

effects observed with layer 5 membranes. It could be that NT-3 is not expressed in layer 5. Obviously, in this case, treatment with the antibodies neutralizing NT-3 would have no influence. Although the exact laminar distribution of NT-3 has not yet been examined in rodents, studies of NT-3 gene expression in cat and primate visual cortex indicate that NT-3 transcripts are detected at very low levels in layer 5 (Lein et al., 1997; Chaudhuri, 1997). It is also possible that in rodents NT-3 is expressed in all layers, but that interactions between cortical axons and some components of layer 5 membranes prevent them responding to NT-3. If this were the case, one might then expect that exogenous application of NT-3 in the cultures should have no effect on branching of axons growing on layer 5 membranes. To address this issue, we examined axonal branch formation of E16 neurons on membranes from different cortical layers in the presence of NT-3 applied to the culture medium. As illustrated in Fig. 7C, when cortical explants were cultured on layers 1-4, 5 and 6 membranes, axonal branching increased in all cases, independently of the laminar origin of the membrane substrata (250 axons examined in two separate experiments). These results indicate that cortical axons are able to respond to exogenous applied NT-3 on a substratum prepared from layer 5 membranes in the same way as on substrata prepared from other cortical layers. Therefore, it seems unlikely that molecular components specifically expressed in layer 5 prevent cortical axons responding to NT-3. It is also possible, however, that NT-3 might be produced by cells in all cortical layers, but that it might not efficiently bind to the cell surface or ECM molecules present in layer 5. If this were the case, then preincubation of membranes from layer 5 with NT-3 should have little effect on axonal branching compared with membranes from other cortical layers. When cortical explants were cultured on membranes from different layers preincubated with NT-3, compared to native membranes axonal branch formation increased significantly on each substratum. However, axonal branching remained twofold lower on layer 5 membranes incubated with NT-3 than on membranes from other cortical layers incubated with

NT-3 (474 axons examined in three separate experiments; Fig. 7D). Thus, the differential effects on axonal branch formation observed on native membranes from different cortical layers remained preserved after preincubating the membranes with NT-3. These results indicate that NT-3 binds less effectively to membrane components present in layer 5 than to membrane components from other cortical layers.

DISCUSSION

During cortical development, axon collateral projections of cortical neurons develop precisely in appropriate layers and almost no branches are emitted in non-target layers. We suggested previously that molecular signals confined in individual laminae provide positional information for the elaboration of layer-specific cortical connections (Castellani and Bolz, 1997a). In the present study, we examined the effects of NT-3 on axonal growth of layers 2/3 and layer 6 neurons. Our findings indicate that NT-3 exerts a dual action on the growth and branching of these two populations of axons. These contrasting effects of NT-3, together with its expression pattern in the developing cortex suggest that NT-3 contributes to the laminar specification of local cortical circuits.

A dual effect of NT-3 in the elaboration of axon collaterals of subsets of cortical neurons

Our findings indicate that application of NT-3 induced axonal sprouting of embryonic layer 6 neurons but decreased axon collateral formation for layers 2/3 neurons. Similarly, a

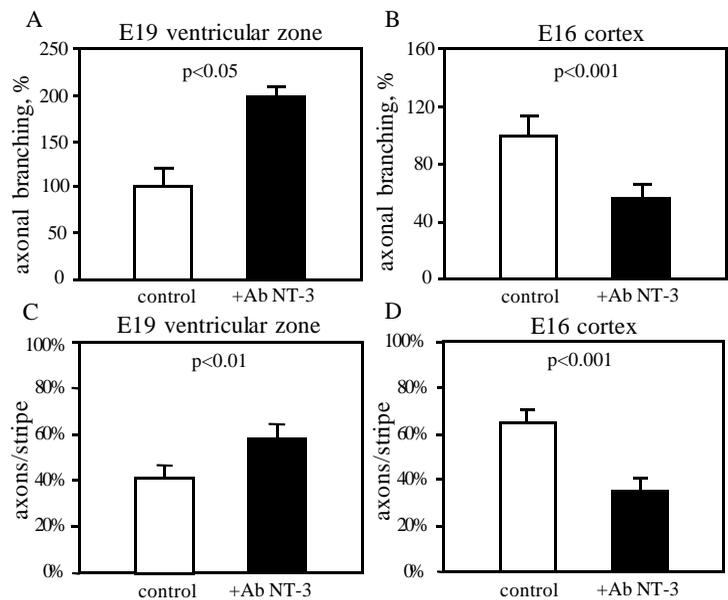


Fig. 5. Quantitative analysis of axonal branching and guidance of cortical explants, cultured on postnatal cortical membranes preincubated with NT-3 antibodies. (A,B) Histograms show that a neutralization of endogenous NT-3 led to a decrease of axonal branching for E16 neurons and to an increase for E19 VZ neurons, indicating that native cortical membranes contain NT-3. (C,D) Membrane stripe assays with cortical explants cultured on alternating lanes of cortical membranes incubated with antibodies directed against NT-3 and untreated membranes. Under these conditions, axons from E16 neurons grew preferentially on control lanes with untreated membranes, whereas E19 VZ axons preferred to grow on membrane lanes treated with the antibodies.

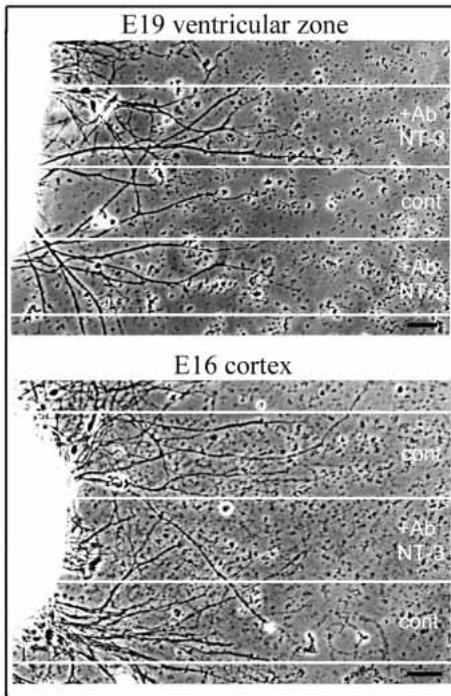


Fig. 6. Phase-contrast photomicrographs illustrating the effect of neutralizing membrane-bound NT-3 in the stripe assays. The lanes are composed of postnatal cortical membranes incubated with NT-3 antibodies (+ Ab-NT-3) alternating with native membranes (cont). E19 VZ axons grew preferentially in the membrane lanes in which endogenous NT-3 was blocked with antibodies, whereas axons from E16 cortex exhibited the opposite behavior. Scale bar, 40 μ m.

promoting and inhibitory effect of the neurotrophin was also observed on axonal elongation of these two populations of neurons. Previous work demonstrated that NT-3 induces sprouting of retinal ganglion cell axons *in vitro* (Inoue and Sanes, 1997) and that local application of NT-3 enhanced axonal branching of corticospinal neurons *in vivo* (Schnell et al., 1994). In the developing cerebral cortex, neurotrophins are likely to play important roles in the remodeling of the cortical connectivity. In the visual cortex, recent works provided several lines of evidence that neurotrophins mediate trophic competitions underlying the segregation of geniculocortical terminals from both eyes into ocular dominance columns (reviewed by Ghosh, 1996; Katz and Shatz, 1996). However, effects of neurotrophins in shaping thalamic terminals remain to be demonstrated. Recently it has been reported that, in cortical slices, endogenous NT-3 promotes dendritic sprouting of layer 6 neurons while it inhibits the dendritic growth of layer 4 neurons. This was the first evidence that NT-3 exerts dual effects on distinct populations of cortical neurons (McAllister et al., 1997). The present results demonstrate that NT-3 also exerts a growth and branching promoting effect on layer 6 axons. Taken together, this suggests that, at least for this population of neurons, common developmental mechanisms may regulate both dendritic and axonal morphology.

Our results with cortical slice cultures indicated that postnatal neurons formed more and longer axon collaterals than embryonic neurons. Under these culture conditions, the morphology of the axon collaterals was heterogeneous, with interstitial extensions of

various length, some tipped with a growth cone. In previous studies, similar types of branches have been observed along callosal, corticospinal and thalamic (Ghosh and Shatz, 1992; Norris and Kalil, 1992; Halloran and Kalil, 1994; Sato et al., 1994; Bastmeyer and O'Leary, 1996). The difference observed in the branching pattern of embryonic and postnatal neurons in slice cultures might reflect maturational changes that occur in the local environment of projecting neurons and/or in the cellular machinery involved in the induction, elongation and stabilization of axon collaterals. Whatever the case, the layer-specific effect of NT-3 on axonal branching was observed in both embryonic and postnatal slice cultures of layer 6 and layers 2/3 neurons. As discussed previously, explants from embryonic tissue do not consist of a pure population of neurons. For instance, E16 explants, in addition to layer 6 neurons, also contain subplate neurons and a portion of neurons destined for layer 5 (Castellani and Bolz, 1997a). However, because axons extending from embryonic explants exhibited a similar behavior to axons from postnatal neurons isolated from individual cortical laminae, we believe that the majority of embryonic axons arise from a layer-specific population of pyramidal neurons. Axons of cortical interneurons very rarely project out of cortical explants (Novak and Bolz, 1993).

Transplantation experiments have shown that young neurons exiting the cell cycle in the ventricular zone are committed to adopting a given laminar position (reviewed by McConnell, 1995). Neurons that have reached their final location in the cortical plate send axonal projections to specific targets, characteristic of the layer that the neurons occupy. Are young postmitotic neurons able to read the environmental cues that would have allowed them, once positioned later on in the layers, to innervate appropriate targets? Our previous work suggested that cortical neurons taken even before exiting the ventricular zone already recognize appropriate signals for the elaboration of their intrinsic projections (Castellani and Bolz, 1997a; Castellani et al., 1998). The present finding provides direct evidence that embryonic cortical neurons destined for layer 6 or layers 2/3 and postnatal neurons taken from the appropriate laminar location respond similarly to an axonal branching signal.

The mechanisms by which NT-3 mediates axonal sprouting are not yet known. NT-3 binds primarily to TrkC receptors and LANR, but it also activates to some extent TrkB and TrkA (Lamballe et al., 1991; Ip et al., 1993; Rodriguez-Tebar et al., 1992). In embryonic rat cortex, high levels of mRNA for both TrkC and TrkB are expressed in the cortical plate and in the ventricular zone (Ernfors et al., 1992; Ringstedt et al., 1993), from which in the present study cortical explants were prepared. The dual effect of NT-3 on cortical neurons destined for distinct layers could therefore be achieved through interactions with these different Trk receptors. Alternatively, because several isoforms of the Trk receptors, including truncated forms that lack the kinase domain, have been described for both TrkC and TrkB (Klein et al., 1990; Middlemas et al., 1991; Valenzuela et al., 1993; Knüsel et al., 1994), the two populations of neurons might express different isoforms of the same Trk receptor, which activate distinct signal transduction cascades. Finally, because in our experiments NT-3 did not influence the number of axons extending from the explants, it is unlikely that the changes observed on axonal elongation and branching are due to a cell survival influence of the neurotrophin. However, we cannot

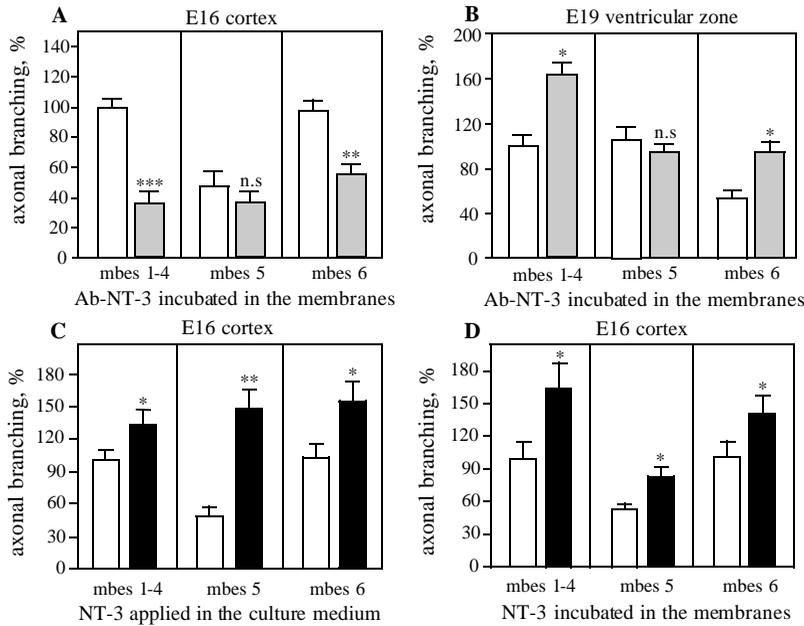


Fig. 7. (A,B) Quantitative analysis of axonal branch formation of cortical neurons, cultured on membrane substrata prepared from isolated layer 6, layer 5 and layers 1-4. (A) On membrane substrata incubated with NT-3 antibodies (black bars), axonal branching of E16 neurons decreased when the explants were cultured on layers 1-4 and layer 6 membranes, compared to untreated membranes (white bars). In contrast, there was no effect on membranes prepared from layer 5. (B) For E19 VZ neurons, the formation of collaterals increased when the axons grew on layers 1-4 and layer 6 membranes incubated with NT-3 antibodies, but not on layer 5 membranes (C) E16 explants were cultured on membrane substrata from isolated cortical layers in the presence (black bars) and the absence (white bars) of NT-3 the culture medium. Under these conditions, NT-3 increased axonal branching independently of the membrane substratum. (D) E16 neurons cultured on membranes from isolated cortical layers, preincubated with NT-3. The increase in axon collateral formation was higher on layers 1-4 and layer 6 membranes, compared to layer 5 membranes.

exclude that some of the effects observed with NT-3 are mediated and/or modulated indirectly through neuronal or glial activities. However, the results with the stripe assays discussed below suggest that at least axonal targeting is mediated by local interactions between growing axons and NT-3.

NT-3 influences axon targeting of cortical neurons

Previous studies have suggested that NT-3 might participate in axonal pathfinding. In postnatal NT-3 mutant mice, sympathetic axons failed to innervate the external ear and the pineal gland. Moreover, injection of NT-3 rescued the innervation pattern, suggesting that target-derived NT-3 might attract growing axons (Elshamy et al., 1996). In slice culture experiments, axonal trajectories of cochleovestibular ganglion cells, which are directed towards the inner ear in control tissue, were strongly disrupted after treatment of the slices with NT-3 antisense oligonucleotides. However, exogenous NT-3 did not restore the target-directed fiber outgrowth (Staecker et al., 1996). Recent in vitro experiments provided more direct evidence that diffusible NT-3 functions as an attractive guidance signal for subsets of embryonic sensory neurons. In these assays, growth cones oriented towards a pipette ejecting NT-3 (Paves and Saarma, 1997). In the present study, we provide evidence that cortical axons are effectively oriented by sharp borders of substratum-bound NT-3 and that endogenous levels of NT-3 present in the developing cortex are able to induce growth cone steering. Thus membrane-bound NT-3 defines permissive territories for the growth of one population of neurons (neurons from E16 cortex) and non-permissive zones for another population cortical neurons (neurons from E19 VZ). Taken together, these data suggests that NT-3 acts as a bifunctional molecule and that it provides both long-range and short-range positional information for the targeting of growing axons.

NT-3 regulates layer-specific axonal sprouting of cortical neurons

Studies using in situ hybridization techniques revealed that NT-

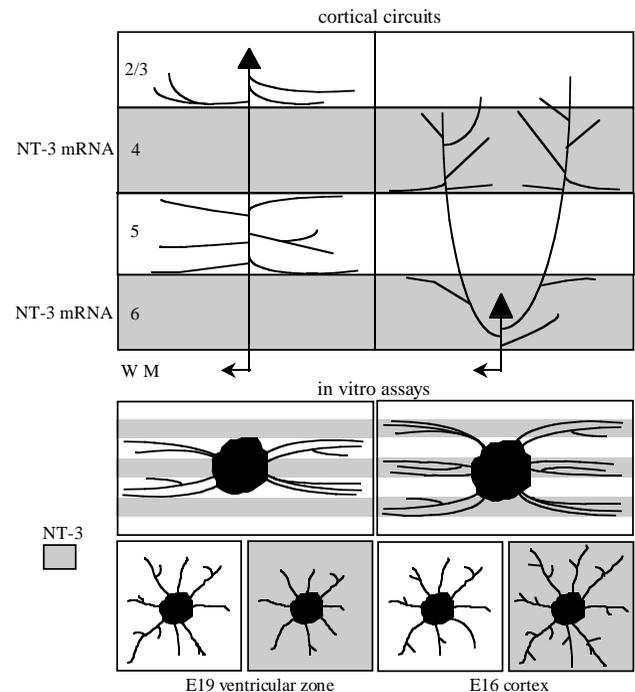


Fig. 8. Model for a possible role of NT-3 in the elaboration of local cortical circuits. (Top) In vivo, axon collaterals of layer 6 neurons originates and extend in layer 6 and layer 4, those cortical layers that express NT-3. Axon collaterals of layers 2/3 neurons exhibit a complementary laminar distribution: collaterals only originate and extend specifically in layers 2/3 and layer 5, that do not express NT-3. (Bottom) In vitro assays from this study indicate that for layer 6 neurons, NT-3 is an attractive axonal guidance signal and that it promotes collateral branch formation. In contrast, for layers 2/3 axons, NT-3 acts as a repellent guidance signal and it inhibits axonal branching. This dual function and the layer-specific expression pattern suggests a role of NT-3 in the assembly of local cortical circuits.

3 is present in the embryonic and postnatal rat cerebral cortex (Maisonpierre et al., 1990; Friedman et al., 1991, 1998; Söderström and Ebendal, 1995) and induces tyrosine phosphorylation of Trk receptors at these developmental stages (Knüsel et al., 1994). The laminar expression pattern of NT-3 in rodents has not yet been worked out in detail. However, it has recently been reported that NT-3 mRNA is selectively detected in layer 4 and layer 6 in the developing cat and primate visual cortex (Lein et al., 1995, 1997; Chaudhuri, 1997). In the present study, application of specific blocking antibodies revealed that postnatal cortical membranes contain NT-3, and that it is efficient to exert branching and guidance effects on cortical axons similar to exogenously applied NT-3. Effects of endogenous NT-3 on axonal branching was observed with membranes from layers 1-4 and 6, but not with layer 5 membranes. These experiments suggest that, in rat cortex, layer 5 contains only low levels of NT-3, as has been found in other mammals. Moreover, the present results also revealed that membrane or ECM components bind NT-3 less efficiently in layer 5 than in layers 1-4 or 6. Interactions of neurotrophins with extracellular matrix molecules, as well as with neuronal and glial surfaces known to express catalytic and non-catalytic forms of Trk receptors, have been described previously (Valenzuela et al., 1993; Cabelli et al., 1996; Gu et al., 1996; for review Korsching, 1993). Thus, even if NT-3 would be produced by cells distributed uniformly throughout the cortex, its differential binding in different cortical laminae would still allow NT-3 to direct layer-specific connections in the developing cortex.

What could be the functional relevance of NT-3 during the elaboration of local cortical circuits? In the developing cortex, intrinsic axon collaterals form and elongate over long distances in distinct laminae, without crossing into adjacent layers (Callaway and Katz, 1990; Galuske and Singer, 1996). Our previous experiments demonstrated that both promoting and repulsive signals expressed in individual cortical layers influence the branching and regulate the layer-specific targeting of distinct populations of cortical axons (Castellani and Bolz, 1997a). The present study demonstrates that NT-3 could be one of the signals that regulate the elaboration of cortical arbors in appropriate layers. As proposed in Fig. 8, layer 6 axons elaborate arbors in response to the branch-inducing properties of NT-3 in layer 4 and layer 6, where this neurotrophin is expressed. In addition, NT-3 also contributes to the targeting of layer 6 collaterals to these layers. On the contrary, because NT-3 has opposing effects on the branching and targeting of layers 2/3 axons, and because NT-3 is expressed in the non-target layers of these neurons, NT-3 could prevent collaterals from layers 2/3 axons arborizing and growing into inappropriate cortical layers. Recently, we have examined the expression pattern and the functional role of Ephrin-A5, a ligand for Eph receptor tyrosine kinases on cortical neurons (Castellani et al., 1998). Ephrin-A5 is selectively expressed in layer 4 and acts as a repellent axonal guidance signal for layers 2/3 neurons, similar to the results described in this study for NT-3. Ephrin-A5 has no guidance effect on layer 6 neurons, but like NT-3 it increases axonal branching for this set of cortical axons. Taken together, these results suggest that molecules involved in the assembly of cortical circuits have a wide and complex range of actions. Such multifunctional wiring molecules appear to act in concert

to specify the intricate network of cortical connections (Bolz and Castellani, 1997).

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