

Accelerated dendritic development of rat cortical pyramidal cells and interneurons after biolistic transfection with BDNF and NT4/5

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Accepted 18 August 2003

Development 130, 5827-5838
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00826

Summary

Neurotrophins are candidate molecules for regulating dendritogenesis. We report here on dendritic growth of rat visual cortex pyramidal and interneurons overexpressing 'brain-derived neurotrophic factor' BDNF and 'neurotrophin 4/5' NT4/5. Neurons in organotypic cultures were transfected with plasmids encoding either 'enhanced green fluorescent protein' EGFP, BDNF/EGFP or NT4/5/EGFP either at the day of birth with analysis at 5 days *in vitro*, or at 5 days *in vitro* with analysis at 10 days *in vitro*.

In pyramidal neurons, both TrkB ligands increased dendritic length and number of segments without affecting maximum branch order and number of primary dendrites. In the early time window, only infragranular neurons were responsive. Neurons in layers II/III became responsive to NT4/5, but not BDNF, during the later time window. BDNF and NT4/5 transfectants at 10 days *in vitro* had still significantly shorter dendrites than adult pyramidal neurons, suggesting a massive growth spurt after 10 days *in vitro*. However, segment numbers were already in the

range of adult neurons. Although this suggested a role for BDNF, long-term activity-deprived, and thus BDNF-deprived, pyramidal cells developed a dendritic complexity not different from neurons in active cultures except for higher spine densities on neurons of layers II/III and VI. Neutralization of endogenous NT4/5 causes shorter and less branched dendrites at 10 days *in vitro* suggesting an essential role for NT4/5. Neutralization of BDNF had no effect. Transfected multipolar interneurons became identifiable during the second time window. Both TrkB ligands significantly increased number of segments and branch order towards the adult state with little effects on dendritic length. The results suggested that early in development BDNF and NT4/5 probably accelerate dendritogenesis in an autocrine fashion. In particular, branch formation was advanced towards the adult pattern in pyramidal cells and interneurons.

Key words: Gene gun, Dendrites, Neurotrophic factors, Autocrine actions, Rodent cortex, Spontaneous activity.

Introduction

The dendritic trees as main entrance port for synaptic information are essential components of neuronal networks and their geometry influences the firing pattern of the neurons (Hume and Purves, 1981; Mainen and Sejnowski, 1996). The mechanisms of dendritogenesis are still under debate. Although cell-autonomous decisions bring forth neuronal polarity (Higgins et al., 1997), a wealth of factors subsequently modulates dendritic growth (McAllister, 2000; Walkley et al., 2000). Transmitters, afferent innervations, neuronal activity and trophic factors acting at the cellular level (McAllister, 2000) represent the mediators of environmental factors like nutrition, sensory experience and enriched environment.

Activity and glutamatergic transmission play a pivotal role depending on receptor status (Rajan and Cline, 1998; McAllister, 2000; Matus, 2000; Inglis et al., 2002). The complexity of basal dendrites of pyramidal neurons increases in the presence of afferents delivering more excitation in co-cultured cortical explants (Baker et al., 1997; Baker and van Pelt, 1997), and spiny stellates in deprived-eye columns extend dendrites into open-eye columns (Kossel et al., 1995). Hippocampal cell dendritogenesis in contrast depends on afferent connectivity rather than activity (Kossel et al., 1997;

Frotscher et al., 2000), whereas activity-deprived CA1 neurons build fewer, but not shorter dendrites (Groc et al., 2002). Synaptic activity evokes further structural changes [e.g. controlling spine formation and synaptic plasticity (Maletic-Savatic et al., 1999; Lendvai et al., 2000; Knott et al., 2002; Frotscher et al., 2000; Thoenen, 2000)]. Transmitters also influence interneuronal morphogenesis (Ross and Porter, 2002; Porter et al., 1999; Jones et al., 2000; Durig and Hornung, 2000).

In pyramidal neurons, exogenous BDNF, NT4/5 (NTF5 – Mouse Genome Informatics) and NT3 (NTF3 – Mouse Genome Informatics), as well as neutralization of endogenous neurotrophins exert layer-specific effects on dendritic growth and branching, with BDNF affecting only active neurons (McAllister, 2000; Baker et al., 1998; Niblock et al., 2000). BDNF also increases dendritic length of hippocampal interneurons (Marty et al., 1996). However, exogenous applications are rather non-physiological in terms of dosages and routes of acquisition. Moreover, the neurochemical markers employed to visualize the interneuronal morphology are often downstream of neurotrophin signaling (Marty et al., 1997; Gorba and Wahle, 1999; Wahle et al., 2000). Furthermore, the neutralization of endogenous TrkB (Ntrk2 –

Mouse Genome Informatics) ligands was usually achieved by TrkB-IgGs, which do not distinguish between BDNF and NT4/5 (McAllister, 2000).

An important issue, therefore, is whether neurotrophins can promote dendritic growth in an autocrine fashion. Horch et al. (Horch et al., 1999) reported that ferret pyramidal neurons overexpressing BDNF develop extra dendrites, as do hippocampal granule cells in BDNF-overexpressing mice (Tolwani et al., 2002) suggesting that BDNF even alters primary neurite patterning. We therefore analyzed in organotypic cultures (OTC) the dendritogenesis of early postnatal rat cortical pyramidal cells and interneurons overexpressing BDNF and NT4/5. Interneurons do not normally express BDNF and the question was whether they could use it. As BDNF is expressed activity dependently in OTC (Gorba and Wahle, 1999), we analyzed the degree of pyramidal cell differentiation in long-term activity-deprived, and thus BDNF-deprived, OTC.

Materials and methods

Organotypic cultures

Roller-type OTC were prepared as described from newborn Long Evans rats (Wirth et al., 1998). Care and use of experimental animals complied with federal German law. In brief, visual cortex was blocked, chopped in 350 μm slices and fixed to a coverslip with a plasma/thrombin coagulate. Cultures were maintained in 0.75 ml of semi-artificial medium (25% Basal Eagle's Medium, 25% horse serum, 50% Gey's Balanced Salt Solution and 1.3 g sucrose) and cultivated in a non-gas incubator for up to 10 days (transfections) or to 30 to 60 days in vitro (DIV; biocytin fillings; see below). Medium was changed every third day.

Expression plasmids

All plasmids were prepared as an endotoxin-free solution (Qiagen, Hilden, Germany). The enhanced 'green fluorescent protein' (EGFP) was used as reporter (pEGFP-N1, CMV-promoter, Clontech, Heidelberg, Germany). The BDNF plasmid (pCMV5-BDNF) was kindly provided by Dr Barde [MPI Martinsried (Leibrock et al., 1989) for BDNF, and Stratagene manual for pCMV]. The NT4/5 plasmid (pCMX-hNT4/5myc) was kindly provided by Dr Yancopoulos (Regeneron, Tarrytown, USA). Both plasmids carry the cytomegalovirus promoter for strong expression in eukaryotic cells. Two independent plasmids were employed rather than bicistronic or chimeric constructs in order to achieve maximal expression, independent processing and targeting and conservation of biological activity of both polypeptides. By imaging immunofluorescence intensities, Horch et al. (Horch et al., 1999) showed that biolistics with similar constructs increases the neurotrophin content in overexpressers within a physiological range.

DNA coating of the gold particles and preparation of the cartridges

The cartridges were prepared as described (Wahle et al., 2000; Wirth and Wahle, 2003). In brief, particle size was 1.5–3.0 μm (Strem Chemicals, Kehl, Germany). The density of gold was 0.2 mg per cartridge. The gold-particles were coated with 1 μg pEGFP-N1 per mg gold. For co-expression of neurotrophic factors and EGFP the same gold-particles were coated with 1 μg pEGFP-N1 per mg gold and 2 μg per mg gold of the expression plasmids for one of the neurotrophic factors. Tissue bombardment was performed as described (Wahle et al., 2000; Wirth and Wahle, 2003) using the hand-held Helios Gene Gun (BioRad, Munich, Germany). The distance from the muzzle to the culture was 1 cm. The helium pressure was 160–200 psi (232×10^{-4} Pa

to 290×10^{-4} Pa). To avoid excitotoxicity by Ca^{2+} influx NMDA receptors were temporarily blocked by adding 3 mM kynurenic acid (Sigma-Aldrich, Deisenhofen, Germany) and 50 μM APV (DL-2-amino-5-phosphonovaleric acid, Sigma-Aldrich, Deisenhofen, Germany) before blasting, and inhibitors were removed 6 hours later. Cortical monocultures were transfected either at the day of explantation (0 DIV=postnatal day 0) or at 5 DIV allowing 5 days for expression. Cultures were harvested at 5 DIV and 10 DIV, respectively.

Detection of expression

EGFP expression reaches its maximum after 24 hours of cultivation and is easily detected with a fluorescence-microscope (FITC filter, excitation at 490 nm/emission at 520 nm). Cultures were fixed in ice-cold 4% phosphate buffered paraformaldehyde (pH 7.4) for 2 hours. Antibody penetration was enhanced with 0.5% Triton-X-100 in TBS for 30 minutes. followed by blocking in 1% bovine serum albumin (Merck, Darmstadt, Germany) and 1% normal goat serum (Dakopatts, Hamburg, Germany) in TBS for 60 minutes. The primary mouse antibody (moxGFP, Clontech, Heidelberg, Germany) was diluted 1:1000 in blocking solution and incubated overnight at 11°C, followed by a 3 hour incubation with a biotinylated secondary antibody (dilution 1:300, Dakopatts, Hamburg, Germany) at room temperature. After several washing steps, OTC were incubated in avidin-biotin-horseradish peroxidase-complex (Dakopatts, Hamburg, Germany), developed with diaminobenzidine and H_2O_2 and mounted with Depex (Sigma-Aldrich, Deisenhofen, Germany). The expression of transfected genes at the mRNA and the protein level was previously shown, and the degree of coexpression of two independent plasmids was 95% (Wirth and Wahle, 2003). Any sampled EGFP-positive transfectant not concurrently overexpressing a neurotrophin would work against our statistics and will not cause false positive results. Both neurotrophin plasmids encode secreted, biologically active peptides because both upregulate neuropeptide Y mRNA in thalamocortical co-cultures after the phenotype restriction [for leukemia inhibitory factor (Wahle et al., 2000); for BDNF and NT4/5 (M.W., unpublished)], but the factors do not accumulate in the medium of transfected cultures to levels, which would upregulate NPY mRNA in untransfected cultures exposed to this medium (not shown).

Pharmacology

Activity deprivation was achieved by addition of 10 mM MgSO_4 to the medium. BDNF or NT4/5 was neutralized by addition of 300 ng/ml of neutralizing antibodies (PeproTech, Rocky Hill, USA) to the medium. Tyrosine kinases were inhibited by 40 nM K252a (Calbiochem/Merck, Bad Soden, Germany). In both experimental conditions, the medium was changed every day from 5 to 10 DIV on.

Generation of cDNA libraries and PCR

Reactions were performed as described (Gorba et al., 1999). In brief, messenger RNA was isolated from at least three OTC for every time point and experimental condition using DynaBeads mRNA Direct Kit (Dyna, Hamburg, Germany). cDNA libraries were synthesized with Sensiscript reverse transcriptase (20 U/ml, Qiagen, Hilden, Germany) at 37°C for 60 minutes. Semiquantitative PCR was performed in a total volume of 50 μl with 5 U/ μl Taq polymerase (Qiagen, Hilden, Germany). Amplified regions for BDNF, NT4/5 and NT3 and glucose-6-phosphate dehydrogenase (GAPDH) were as described, GAPDH was chosen as an activity-independent standard (Gorba et al., 1999). The number of cycles was kept in the linear range determined for each product. The amplicates were densitometrically analysed with the Eagle Eye system (Stratagene, Amsterdam, The Netherlands) and normalized to GAPDH. Normalized values from a total of 10 PCR reactions from two independent cDNA libraries were used to construct the graphs with s.e.m.

Biocytin injections

Pyramidal cells and interneurons of spontaneously active and

pyramidal cells of chronically activity-deprived (with 10 mM MgSO₄ in the medium) OTC aged 30 to 60 DIV (summarized here as 'adult') were analyzed. Activity deprivation was initiated at 3 DIV and lasted until the time of analysis. Cells were filled with biocytin after intracellular electrophysiological recording followed by histochemical staining (Klostermann and Wahle, 1999; Gorba et al., 1999). Electrophysiological recording confirmed the lack of bioelectric activity in OTC with 10 mM MgSO₄ in the medium.

Analysis

Pyramidal neurons from layers II/III, V and VI and were reconstructed with a Eutectics Neuron Tracing system (Raleigh, USA) at 1000× magnification from cultures cut strictly perpendicular to the cortical surface as indicated by apical dendrites of layer V cells connecting to layer I, by transfected glia cells with radial processes extending into supragranular layers, by the absence of transfected cells with obviously truncated apical dendrites and by the presence of supragranular pyramidal cell axons descending perpendicularly to the white matter.

All pyramidal cells included here resided fairly isolated in order to trace all the processes of only one neuron. They had axons with a clear origin at the soma or a proximal dendrite. Supragranular neurons were included when apical dendrites extended into layer I. Layer VI neurons were included when apical dendrites ended in middle layers. Our sample of layer V neurons included only neurons with apical dendrites in layer I (corticotectal neurons). Interneurons were sampled from layers II/III and V/VI in the later time window and were pooled. We restricted the analysis to multipolar cells with smooth or sparsely spinous dendrites and locally branching axons this way enriching for presumptive basket neurons. Interneurons with a polarized appearance were excluded, because immature Martinotti or bi-tufted neurons were not always distinguishable from immature pyramidal cells.

Total dendritic length, length of apical and basal dendrites, number of segments, maximum branch order, number of primary dendrites were determined for neurons of layers II/III, V and VI. For the biocytin-labeled neurons the spine density was determined for the entire dendrite and is given in the Fig. 7 as density per 100 μm. Data

are presented as means with s.e.m. For statistical analysis, the non-parametric Mann-Whitney U-test was performed since often the data did not pass the tests for equal variance and normality (Kolmogorov-Smirnov-test for normality, both tests applied via SigmaStat program, SPSS). For multiple testing the α -value was corrected according to Holm (Holm, 1979).

Results

The early time window: infragranular pyramidal neurons respond

For pyramidal neurons, the mean number of primary dendrites and the maximum branch order was not different between EGFP control transfectants and neurotrophin-transfectants. There was no evidence for exuberant numbers of short perisomatic dendrites selectively after transfection with BDNF (Fig. 1). Somata with this appearance occurred rarely in all three groups of transfectants, suggesting a response to damage as a cause.

Layer II/III neurons were very immature. Many transfectants displayed the morphology of migrating neurons (small elongated soma, bipolar unbranched neurites); they were not sampled here. The more mature transfectants sampled typically had one already branching apical and two to three basal dendrites (Fig. 1). The maximum branch order was four or five. Mean apical dendritic length in the three groups of transfectants was 400 μm, and mean basal dendritic length was 80 μm (Fig. 2; see also Table 1 for total basal dendritic length and total number of basal dendritic segments).

Layer V pyramidal neurons (Figs 1, 2) had larger triangular somata, an apical dendrites in layer I and a mean of three basal dendrites. The maximum branch order was six. The apical dendrites of EGFP and NT4/5 transfectants were 600-700 μm in length. Only BDNF showed a non-significant trend to increase apical dendritic length (Fig. 2). Apical dendritic segments varied from 12-15 in EGFP and NT4/5 transfectants to about 20 in BDNF transfectants (Fig. 2), but these differences were not significant. However, BDNF significantly increased the length of basal dendrites ($P < 0.01$) and the number of segments ($P < 0.05$; Fig. 2).

Layer VI pyramidal neurons had one apical dendrite and three or four basal dendrites (Figs 1, 2) and a maximum branch order of 4-6. BDNF and NT4/5 transfectants displayed significantly longer apical dendrites ($P < 0.05$ for both) and basal dendrites (BDNF: $P < 0.05$; NT4/5: $P < 0.01$; Fig. 2). Furthermore, the mean number of apical segments was significantly increased by BDNF and NT4/5 when compared with EGFP control (BDNF: $P < 0.05$; NT4/5: $P < 0.01$; Fig. 2). Basal segment numbers were only increased by NT4/5 ($P < 0.05$).

Thus, both neurotrophins increased dendritic length and branching during the early time window with NT4/5 appearing more effective than BDNF, but effects were only in infragranular layers.

The later time window: supragranular neurons become responsive

Overall, the 10 DIV neurons were clearly more mature than 5 DIV neurons (Fig. 1) indicating the

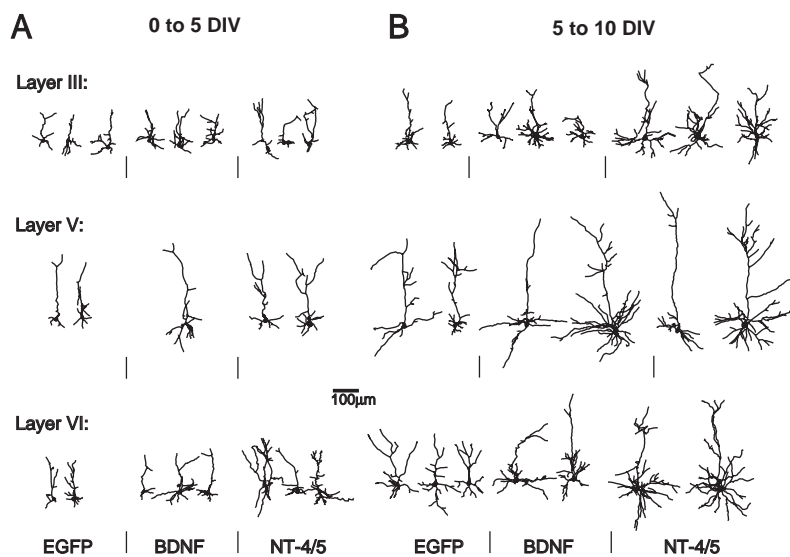


Fig. 1. Representative pyramidal neurons from layers II/III, V and VI transfected with EGFP, BDNF/EGFP and NT4/5/EGFP. (A) Neurons of the early time window, DIV 0-5. Only layer VI neurons displayed more profuse dendritic trees. (B) Neurons of the later time window, DIV 5-10. Now BDNF and NT4/5 strongly accelerated the development of apical and basal dendrites. Note that neurotrophin transfectants at 5 DIV appear quite similar to EGFP control transfectants at 10 DIV.

Table 1. Dendritic length and segment numbers in culture

	5 DIV EGFP	10 DIV EGFP	10 DIV BDNF	10 DIV NT-4/5	Adult, active OTC	From literature
Total basal dendritic length (μm ; mean \pm s.e.m.)						
Layer II/III	186 \pm 23	423 \pm 48	575 \pm 75	574 \pm 54	2365 \pm 325	P10: \sim 300 μm^* , Adult: \sim 1800 μm^* P30/60: \sim 1400 μm^\dagger , Adult: 2630 \pm 500 μm^\ddagger
Layer V	275 \pm 39	715 \pm 118	1354 \pm 271	702 \pm 53	2640 \pm 475	P10: \sim 800 μm^* , Adult: \sim 2220 μm^* P30/60: \sim 1100 μm^\dagger
Layer VI	243 \pm 24	447 \pm 59	914 \pm 265	1238 \pm 95	2565 \pm 318	No data available
Total number of basal dendritic segments (\pm s.e.m.) and tips						
Layer II/III	6.8 \pm 1.3	12.4 \pm 1.2	21.8 \pm 2.4	23 \pm 2.2	56.6 \pm 7.9 tips: 29 \pm 6.0	Tips: 27 ‡ , 29.2 \pm 7.5 §
Layer V	9.6 \pm 3.2	22.1 \pm 4.3	30.3 \pm 5.6	34.8 \pm 3.4	42.7 \pm 6.7 tips: 27 \pm 5.3	Tips: 34.6 \pm 5.7 §
Layer VI	11.9 \pm 1.7	15.6 \pm 1.5	27.8 \pm 4.2	24.0 \pm 2.2	39.9 \pm 4.0 tips: 23.4 \pm 3.8	No data available

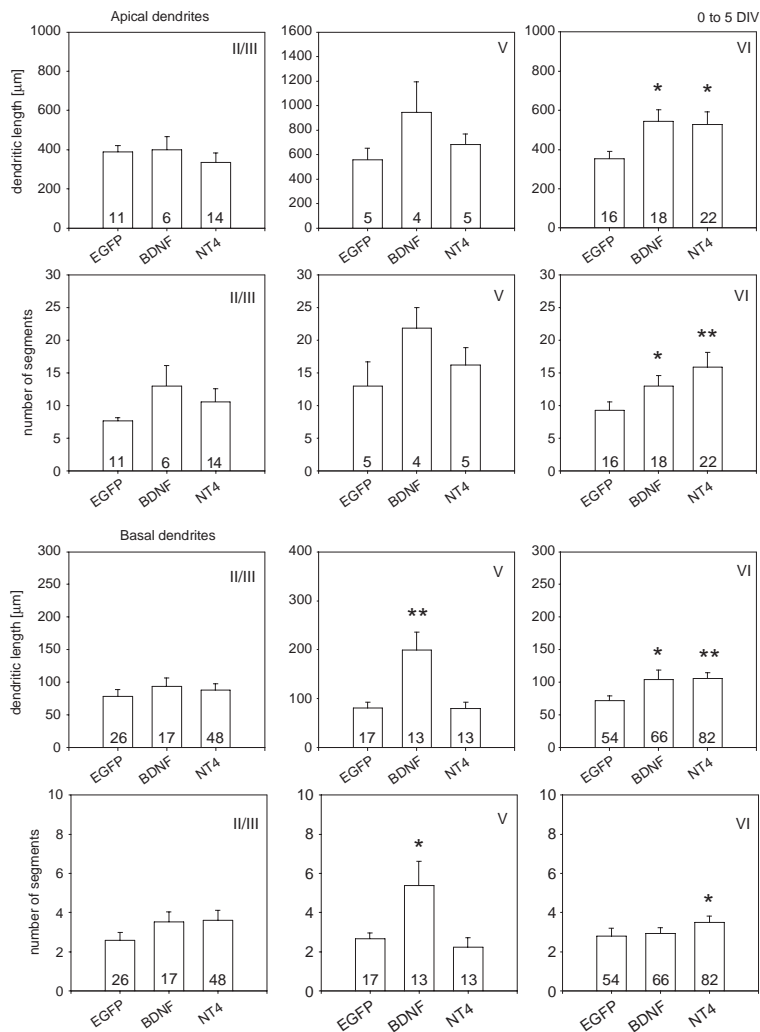
Total length of basal dendrites (mean in $\mu\text{m}\pm$ s.e.m.) and total number of basal dendritic segments (mean \pm s.e.m.) in the conditions indicated in comparison to adult pyramidal neurons in vivo. Lengths in vivo may be shorter than in OTC because of sectioning.

*Uylings et al., 1990.

† Juraska, 1982.

‡ Schröder and Luhmann, 1997.

§ Larkman and Mason, 1990.



expected progressive differentiation in OTC with time (compare 5 with 10 DIV in Table 1; Fig. 3). In layers II/III, the length of apical dendrites of EGFP control was still around 400 μm . Compared with 5 DIV EGFP transfectants, the basal dendritic length of 10 DIV EGFP transfectants had almost doubled. Total basal dendritic length was in the range previously reported for P10 (Table 1).

NT4/5 transfectants now displayed significant longer apical dendrites ($P<0.01$; Fig. 3) and basal dendrites ($P<0.05$; Fig. 3) with a corresponding significant increase in segment number (apical: $P<0.05$; basal: $P<0.01$; Fig. 6). BDNF failed to elicit significant responses.

Layer V neurons were clearly advanced compared with the early time window (Fig. 3). Apical dendritic length had doubled in EGFP transfectants compared to EGFP transfectants at 5 DIV. There was no additional increase in the neurotrophin transfectants (Fig. 2). For basal dendrites, BDNF significantly increased the length ($P<0.05$; Fig. 3), and both TrkB ligands significantly increased the number of segments ($P<0.05$; Fig. 3).

In layer VI, neurons (Fig. 3) NT4/5 significantly increased the length of apical ($P<0.05$) and basal dendrites ($P<0.01$). BDNF had weak effects on apical

Fig. 2. The early time window: quantification of dendritic length and number of segments for apical and basal dendrites of pyramidal neurons in layers II/III, V and VI after overexpression of EGFP, BDNF/EGFP and NT4/5/EGFP. *= $P<0.05$; **= $P<0.01$, Mann-Whitney-U-tests versus EGFP control. The numbers in the bars represent the number of dendrites analyzed. For apical dendrites they equal the number of neurons analyzed.

dendrites but significantly increased the length of basal dendrites ($P < 0.01$; Fig. 3). Segment numbers of basal and apical dendrites were significantly increased by BDNF (apical, $P < 0.05$; basal, $P < 0.05$) and NT4/5 (apical, $P < 0.05$; basal, $P < 0.05$; Fig. 3).

Thus, both TrkB ligands strongly promoted dendritic maturation of infragranular neurons and supragranular neurons became responsive to NT4/5.

Comparison to adult pyramidal cells in vitro

A central question was whether the structural changes evoked by the factors were really advancing differentiation towards an adult state. We therefore analyzed a set of biocytin-filled pyramidal cells obtained previously in spontaneously active adult OTC (30–60 DIV of age) (Klostermann and Wahle, 1999) with the assumption that they represent an end point of maturation in OTC. Indeed, their morphometric parameters were quite similar to in vivo pyramidal cells in the adult cortex. The neurons displayed a mean of 5 primary dendrites, which was in line with previously published data (Schröder and Luhmann, 1997; Miller, 1981; Juraska, 1982). The total length of basal dendrites of layer II/III and of layer V pyramidal neurons was about 2300 μm and 2600 μm , respectively. Length and the number of dendritic tips were in the range of adult pyramidal cells in vivo (Table 1). This suggested that pyramidal cells in spontaneously active OTC acquire a degree of differentiation quite similar to in vivo pyramidal neurons in the adult cortex.

When comparing dendritic length and complexity of transfectants at 10 DIV to adult neurons there was a clear difference between the two sets of cells. Apical and basal dendrites at 10 DIV were shorter (Table 1; also compare Fig. 3 with Fig. 7). Even the most advanced transfectants at 10 DIV were still immature when compared with adult neurons aged 30–60 DIV suggesting major growth spurts after day 10.

The interesting parameter was basal dendritic segment number (Table 1). Not surprising, layer II/III neurons had highly significantly fewer segments at 10 DIV compared with adult. Although the neurotrophins doubled the total basal dendritic segment number, they failed to evoke the adult dimensions. By contrast, total basal dendritic segment numbers of neurotrophin transfectants in layer V and layer VI were fairly close to the adult pattern. Statistically, layer V cells were no longer different from adult (BDNF, $P = 0.56$; NT4/5, $P = 0.72$), whereas in layer VI the BDNF transfectants were close to adult ($P = 0.06$) while NT4/5 transfectants still were significantly smaller than adult ($P < 0.01$). Together this suggested that the early neurotrophin transfections precociously advance branch formation and evoke segment numbers, which at 10 DIV were in the range of adult pyramidal neurons.

Interneuronal dendritic complexity increased with overexpression of TrkB ligands

Multipolar interneurons (Fig. 4) became recognizable during the first postnatal week in vivo and in OTC (Obst and Wahle,

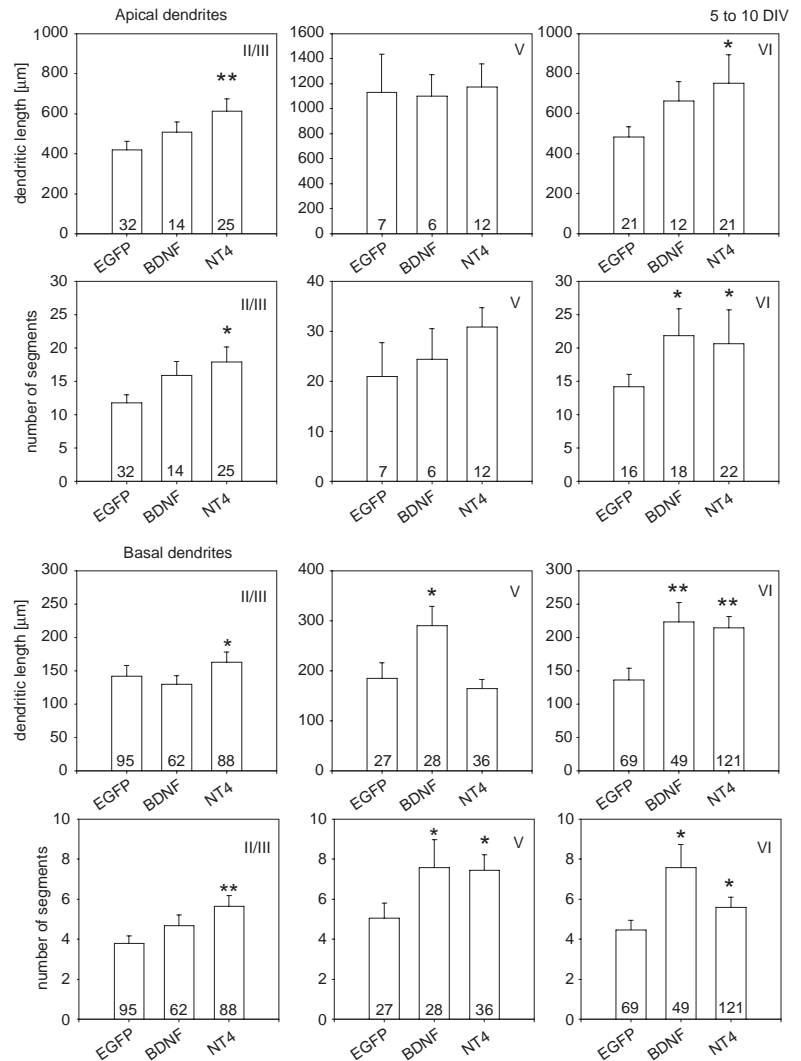


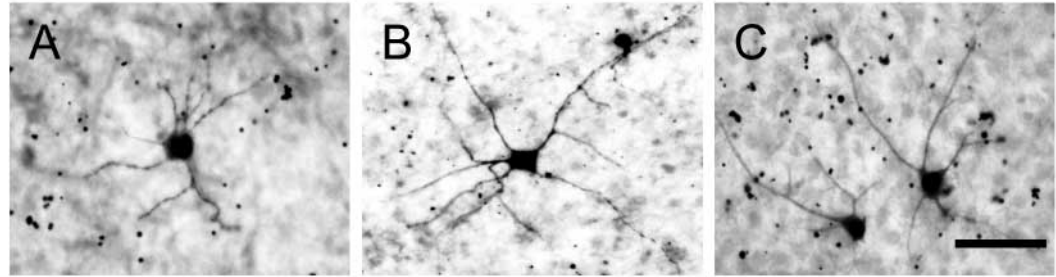
Fig. 3. The later time window: quantification of dendritic length and number of segments for apical and basal dendrites of pyramidal neurons in layers II/III, V and VI after overexpression of EGFP, BDNF/EGFP and NT4/5/EGFP.

*= $P < 0.05$; **= $P < 0.01$, Mann-Whitney-U-tests versus EGFP control. The numbers in the bars represent the number of dendrites analyzed. For apical dendrites, they equal the number of neurons analyzed. See also Table 1 for total basal dendritic length and segment numbers.

1995). Dendritic length and complexity steadily increases during the second and third week (Mathers, 1979; Uylings et al., 1990; Miller, 1986), which overlaps with our 5–10 DIV transfection period. Fig. 3 shows the interneuronal data in direct comparison to adult multipolar interneurons in vitro.

First, there were no differences in the number of primary dendrites between the three conditions [Fig. 5; about five per cell; in line previous reports (Miller, 1986; Uylings et al., 1990)]. At 10 DIV the mean dendritic length of EGFP interneurons was 244 μm . BDNF- and NT4/5-overexpressing interneurons had a mean dendritic length of 304 μm and 329 μm , respectively. Although the dendritic length increased noticeable under overexpression of both TrkB ligands, these differences were not significant (Fig. 5). As we selected only multipolar neurons with non-polarized dendritic fields, we calculated the total dendritic length. This was now significantly

Fig. 4. Photomicrographs of representative multipolar interneurons after transfection with (A) EGFP, (B) BDNF/EGFP and (C) NT4/5/EGFP. Scale bar: 100 μ m.



increased with BDNF and NT4/5 compared with EGFP (both $P < 0.05$). The dendritic complexity increased significantly from 6.1 segments per dendrite in EPFG interneurons to 9.8 in BDNF and 10.5 in NT4/5 overexpressing interneurons (Fig. 5). Accordingly, the maximum branch order was highly significantly increased from a mean of 5.2 in EGFP controls to 8.1 in BDNF- and 7.5 in NT4/5-overexpressing interneurons ($P > 0.01$). Together this suggested that both TrkB ligands have a weak effect on dendritic elongation, but strongly promote branch formation.

Comparison to the adult interneurons in vitro

We selected the dendritic parameters of multipolar fast-spiking interneurons from layers II/III and V/VI recorded and biocytin-filled at ages of 30-60 DIV (Klostermann and Wahle, 1999) assuming that they represent the adult stage. Reports on interneuronal morphometry in vivo exist only for layer IV interneurons (Uylings et al., 1990). The dendritic length and complexity of our in vitro sample was larger when compared with published layer IV interneurons in vivo because, in particular, infragranular interneurons are larger than those from middle layers (Feldman and Peters, 1978; Meyer, 1983).

Compared with our adult interneurons in vitro, the transfectants were not different with respect to the number of primary dendrites (Fig. 5). The 10 DIV neurotrophin transfectants had much shorter dendrites and total dendritic length ($P < 0.0001$). The mean total dendritic length of the EGFP transfectants was about 1000 μ m, BDNF transfectants had 1600 μ m, and NT4/5 transfectants had 1400 μ m at 10 DIV as compared with about 4000 μ m of adult interneurons (Fig.

5). However, maximum branch order and number of segments were statistically no longer different from adult (Fig. 5). This suggested that the TrkB ligands precociously evoke the adult number of segments, whereas the adult length develops after 10 DIV.

Activity blockade does not prevent dendritic maturation of pyramidal neurons

The study so far revealed that TrkB ligands secreted by the transfectants accelerated dendritogenesis in some aspects close to the adult in vitro pattern. The important question now concerned the role of endogenous factors. As BDNF is activity-dependently synthesized and released (Androutsellis-Theotokis et al., 1996; West et al., 2001; Tao-Cheng et al., 2002; Balkowiec and Katz, 2002) (for a review, see Lessmann et al., 2003) we took advantage of activity-deprived OTC in which the endogenous BDNF mRNA expression, but not the NT4/5 and NT3 mRNA expression is strongly reduced (Gorba et al., 1999; Ichisaka et al., 2003). Furthermore, the activity-dependent component of neurotrophin release should be eliminated. If BDNF were essential, pyramidal neurons from activity-deprived and thus BDNF-deprived OTC should display less differentiated dendrites.

We compared 30-60 DIV adult pyramidal neurons of layers II/III, V and VI in chronically activity-deprived OTC to adult neurons from active OTC. All were pyramidal by morphological and electrophysiological classification (Gorba et al., 1999) with polarized dendritic trees covered by spines (Fig. 6A-C for active and Fig. 6D-F for activity-deprived pyramidal neurons). In fact, the deprived neurons were surprisingly well differentiated (compare Fig. 6A-D). Neither apical nor basal dendritic length differed significantly (Fig. 7). In layers II/III the number of primary dendrites (a mean of 5), maximum branch order (of 10) and number of segments per dendrite of pyramidal neurons from activity-deprived OTC were not different from those of active OTC (Fig. 7). Layer V pyramidal neurons were unchanged. In layer VI pyramidal cells, the number of primary

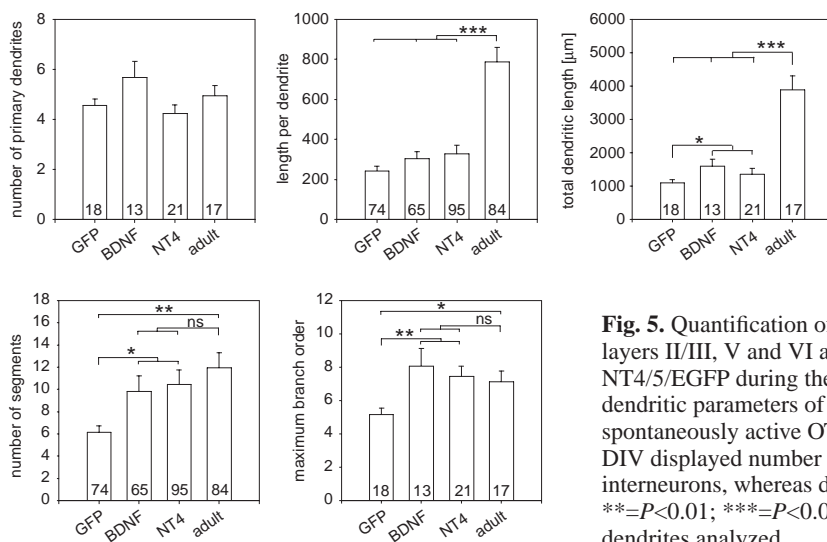


Fig. 5. Quantification of dendritic parameters of multipolar interneurons from layers II/III, V and VI after overexpression of EGFP, BDNF/EGFP and NT4/5/EGFP during the later time window, DIV 5-10. For direct comparison, dendritic parameters of biocytin-filled multipolar interneurons from adult spontaneously active OTC were included. The neurotrophin transfectants at 10 DIV displayed number of segments and maximum branch orders close to adult interneurons, whereas dendritic length was still far from being mature. *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$. The numbers in the bars represent the number of dendrites analyzed.

dendrites (a mean of 5), the maximum branch order (of 10) and the number of segments per dendrite were not different.

The major difference was the spine density (compare Fig. 6B,C with 6E,F; Fig. 7). Active neurons had about 15-30 spines

per 100 μm dendrite which was much lower than reported for adult pyramidal neurons in the cortex in vivo [data extracted from other (Miller, 1981; Juraska, 1982; Schröder and Luhmann, 1997; Kolb et al., 1997) and calculated per 100 μm dendrite; layers II/III basal, 65-110; layers II/III apical, 60-102; layer V basal, 60-100; layer V apical, 60-120 spines). By contrast, apical and basal dendrites of activity-deprived neurons from layers II/III and layer VI, but not layer V, had significantly increased spine densities (all $P < 0.01$; Fig. 7), which, however, were still lower than densities reported for pyramidal neurons in vivo.

NT4/5 but not BDNF plays a physiological role for pyramidal cell dendritogenesis

We have shown that overexpressed BDNF and NT4/5 accelerate dendritogenesis. However, neurons grown under activity deprivation and therefore in the absence of BDNF showed surprisingly 'normal' dendrites. Thus, the question is whether BDNF or NT4/5 is more important for dendritogenesis. We focused on development of dendritic length and segment number of layer VI pyramidal neurons because they displayed a strong response to both TrkB ligands during the second time window.

To exclude the possibility that activity-deprived pyramidal neurons from OTC aged 30 to 60 DIV just compensated for the lack of BDNF simply by having enough time for dendritogenesis, we analyzed neurons activity-deprived from 5-10 DIV. The dendritic length and the number of segments were not significantly different from spontaneously active controls (Fig. 8) suggesting that short-term deprivation of activity and thus of BDNF does not impair dendritogenesis.

To directly prove that BDNF is not essential for dendritogenesis we neutralized the endogenous BDNF with antibodies. Neither dendritic length nor segment numbers differed from control (Fig. 8). By contrast, the neutralization of endogenous NT4/5 significantly decreased dendritic length and complexity (Fig. 8) suggesting that NT4/5 is an essential endogenous factor for dendritogenesis in this time window.

Both factors failed to promote dendritic growth in transfected layer VI pyramidal neurons in the presence of K252a (Fig. 8) suggesting that the action of overexpressed BDNF and NT4/5 depends on Trk receptors. However, EGFP-transfected neurons had significantly smaller and less complex dendrites in the presence of K252a than did untreated EGFP control neurons (Fig. 8) suggesting that endogenous neurotrophins acting via Trk receptors contribute to dendritic growth.

As expected, the BDNF mRNA expression remained very low in activity-deprived OTC (Fig. 9A). By contrast, activity-deprived OTC displayed a much higher expression of NT4/5

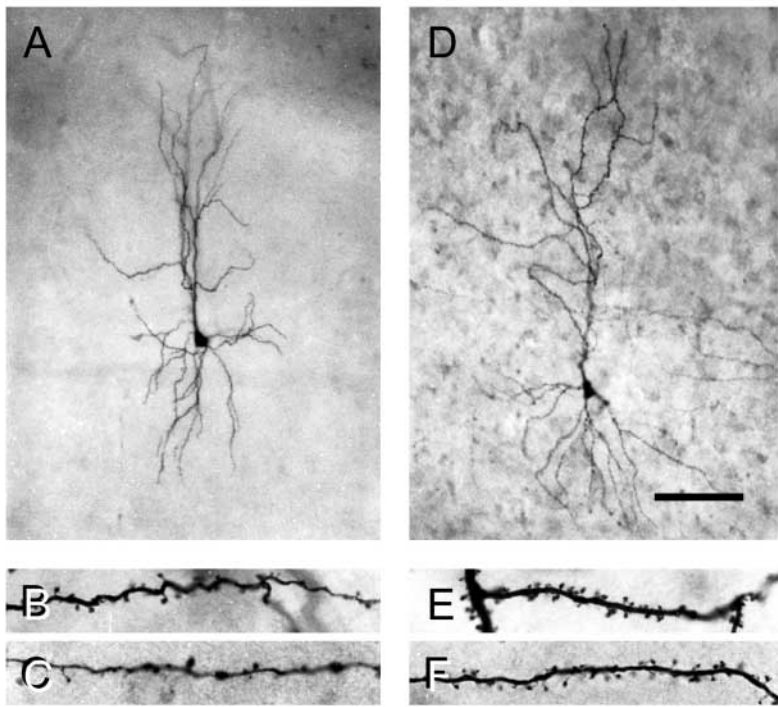


Fig. 6. Representative pyramidal cells of layers II/III from adult OTC. (A-C) Neuron and high magnifications of spiny dendrites from active OTC. (D-F) Neuron and high magnifications of spiny dendrites from activity-deprived OTC. Scale bar: 150 μm .

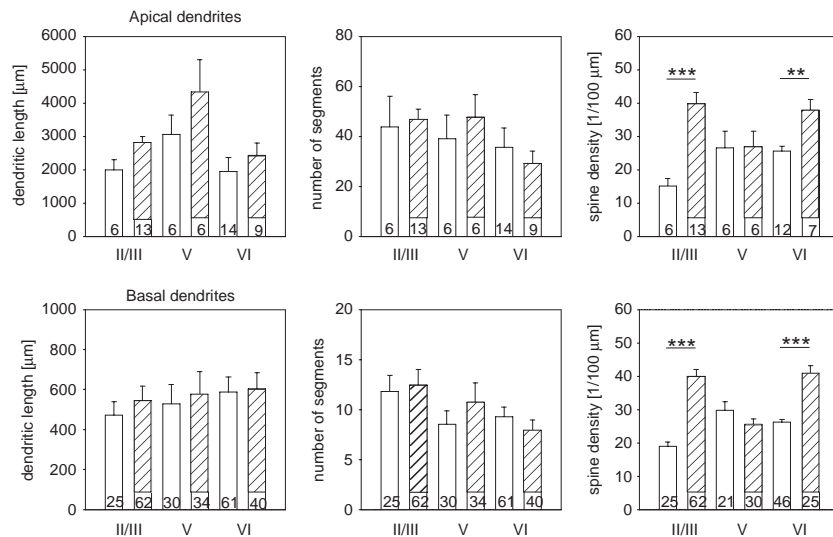


Fig. 7. Quantification of dendritic parameters of adult pyramidal neurons of layers II/III, V and VI from active (white bars) and activity-deprived (hatched bars) OTC. Neither length nor segments numbers of apical and basal dendrites differed significantly. By contrast, spine density was significantly increased on apical and basal dendrites of pyramidal cells from layers II/III and VI. $** = P < 0.01$; $*** = P < 0.001$. The numbers in the bars represent the number of dendrites analyzed. For apical dendrites, they equal the number of neurons analyzed.

mRNA and to a lesser extent also of NT3 mRNA at 10 and 20 DIV, but no longer at 45 DIV (Fig. 9B,C) suggesting that NT4/5 and NT3 are present in higher amounts during the main period of dendritic development. This supported the view that the dendritic differentiation in activity-deprived OTC is primarily governed by NT4/5.

NT3 is released via a constitutive and therefore activity-independent pathway (for a review, see Lessmann et al., 2003). To prove the activity-independent release and action for NT4/5, we analyzed NT4/5 overexpressing layer VI pyramidal neurons in activity-deprived OTC. In fact, the overexpressed NT4/5 significantly increased the dendritic length and complexity in the absence of electrical activity (Fig. 8), while overexpressed BDNF failed (Fig. 8).

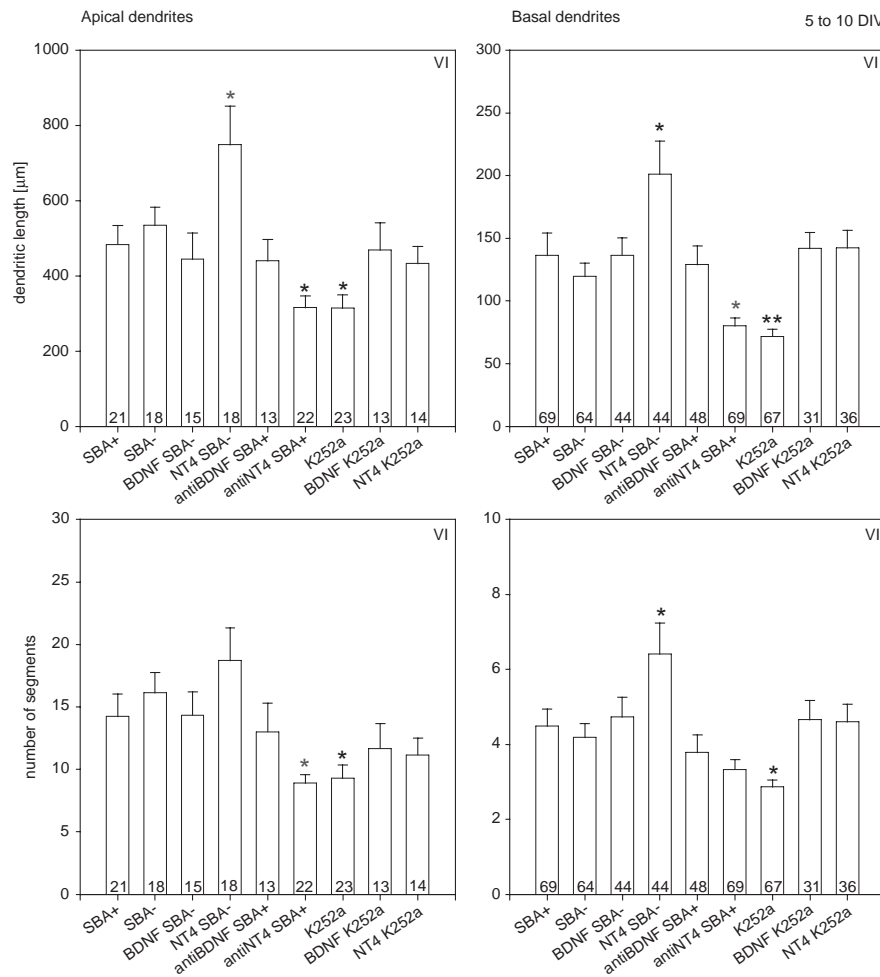


Fig. 8. NT4/5 but not BDNF is essential for dendritogenesis. Apical and basal dendritic length and number of segments were quantified for pyramidal neurons in layer VI in the following conditions (indicated on the abscissa): SBA+, EGFP transfectants in active OTC; SBA-, EGFP transfectants in deprived OTC; BDNF SBA-, BDNF transfectants in deprived OTC; NT4 SBA-, NT4/5 transfectants in deprived OTC; anti-BDNF SBA+, EGFP transfectants in active OTC treated with neutralizing antibodies against BDNF from 5-10 DIV; antiNT4 SBA+, EGFP transfectants in active OTC treated with neutralizing antibodies against NT4/5 from 5-10 DIV; K252a, EGFP transfectants in active OTC treated with K252a from 5-10 DIV; BDNF K252a, BDNF transfectants in active OTC treated with K252a from 5-10 DIV; NT4 K252a, NT4/5 transfectants in active OTC treated with K252a from 5-10 DIV. The numbers in the bars represent the number of dendrites analyzed. For apical dendrites they equal the number of neurons analyzed. *= $P < 0.05$; **= $P < 0.01$, Mann-Whitney-U-tests versus SBA+ condition.

To summarize, dendritic maturation of pyramidal neurons and multipolar nonpyramidal neurons overexpressing BDNF and NT4/5 was accelerated in a layer- and time-dependent manner, suggesting an autocrine role for the TrkB ligands. However, pyramidal cells deprived of activity and of endogenous BDNF differentiate with high fidelity probably owing to an activity-independent action of NT4/5.

Discussion

BDNF and NT4/5 accelerate the dendritic differentiation of pyramidal neurons

Both factors increased the length and complexity of pyramidal cell dendrites. Their effectiveness depended primarily on developmental stage. During the early time window infragranular neurons are post migratory, layers V/VI are segregated, and cells responded to both TrkB ligands. Cells in supragranular layers were not yet responsive. Many are immature and still migrating and layers are not yet segregated. During the later time window, pyramidal neurons were more mature. Effects that existed as trends or had weak significances in the early time window became prominent. Infragranular neurons now strongly reacted to both factors and supragranular neurons responded to NT4/5.

Neurotrophin transfectants displayed at 5 DIV a degree of differentiation similar to control transfectants at 10 DIV (see Fig. 1). Neurotrophin transfectants at 10 DIV displayed an almost adult degree of branching, but not yet length. The neurotrophins apparently accelerated dendritogenesis in a step-by-step manner without evoking a precocious hypertrophy. A gradual acceleration was also observed for somatic development of pyramidal cells in mice postnatally overexpressing activated p21Ras acting as an intracellular neurotrophin, and somatic hypertrophy was persisting with adult neurons having thicker and more branched dendrites (Heumann et al., 2000) (P.W., unpublished). Similar to some other G proteins (Threadgill et al., 1997), activated Ras thus mimics the effects reported here suggesting that they are evoked by autocrine neurotrophin signaling known to occur in neurons (Miranda et al., 1993; Acheson and Lindsay, 1996; Giehl, 2001). Paracrine actions cannot be excluded, but are considered unlikely for the following reasons. It would imply that two neurotrophin overexpressers interact reciprocally to drive each other's differentiation. When considering an axonal route of delivery one must take into account that the intracortical axonal connections are fairly underdeveloped at

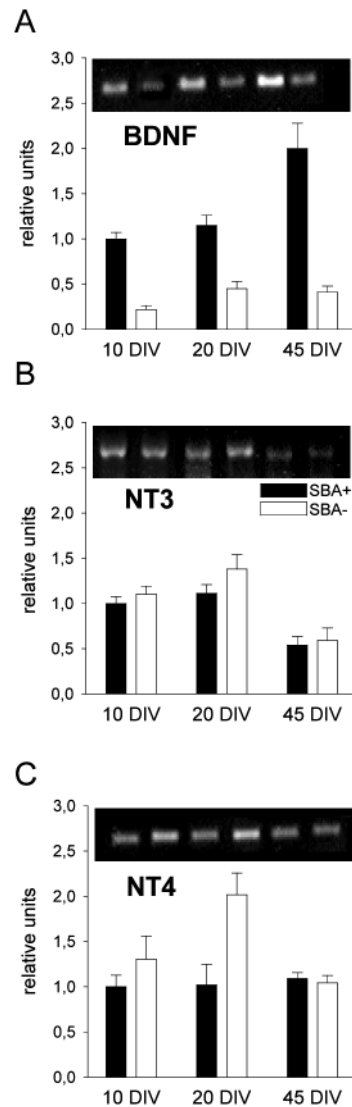


Fig. 9. Developmental mRNA expression of BDNF, NT3 and NT4/5 in active (SBA+, black bars) and activity-deprived (SBA-, white bars) OTC. (A) BDNF mRNA expression increased with age in active OTC, but remained low in deprived OTC. (B) NT3 mRNA expression was slightly upregulated in young deprived OTC and decreased until 45 DIV. (C) NT4/5 mRNA expression was strongly upregulated in 10 and 20 DIV activity-deprived OTC. The expression levels of the three mRNAs in the 10 DIV SBA+ condition were set to 1.

the ages investigated (DIV 5 and DIV10). When considering a somatodendritic release, the partner cells must be in very close by because Horch and Katz (Horch and Katz, 2002) have shown that BDNF released from overexpressors can modify dendrites only within a 4.5 μm distance. Yet, our overexpressors had been selected for reconstruction by their fairly isolated position, which is a prerequisite for tracing all the fine processes of only one neuron.

Despite methodical differences and the difficulty to extrapolate developmental time-course between rat and ferret, our results are comparable with studies by McAllister et al. (McAllister et al., 1995; McAllister et al., 1996; McAllister et al., 1997). The authors found strong effects of exogenous BDNF and NT4/5 for infragranular pyramidal neurons, although in contrast to ferret, rat layer V neurons strongly responded to BDNF and layer VI neurons responded to both factors, without evidence for a growth-inhibitory BDNF action. We further showed that the most immature layer II/III neurons were still unresponsive. As exogenous BDNF affects only active neurons as has been shown for its dendritogenic (McAllister et al., 1996) and phenotype-promoting actions

(Marty et al., 1997; Wirth et al., 1998), the lack of BDNF effects in, for example, 5-10 DIV supragranular transfectants appeared due to low activity or a failure to activity-dependently release overexpressed BDNF. Niblock et al. (Niblock et al., 2002) found little effects of exogenous BDNF in P11 rat cortical layer II pyramidal cells, which had just finished migration. Only basal dendritic branching increases, possibly owing to the high doses used. Apparently, very immature neurons fail to react to BDNF either due to a lack of local activity or an immaturity of intrinsic excitability. An intriguing possibility is that the onset of BDNF action correlates with the acquisition of specific Na^+ channel function (Kafitz et al., 1999). By contrast, the effectiveness of NT4/5 is due to its activity-independent action (Wirth et al., 1998), and our results strongly suggest an activity-independent release of NT4/5.

BDNF and NT4/5 failed to induce primary dendrites

In contrast to studies by McAllister et al. (McAllister et al., 1995) and Horch et al. (Horch et al., 1999) in ferret, we did not find dramatic increases in primary dendrites. This could be due to species differences. Rodent neurons are for instance less affected by gangliosides, which evoke aberrant dendrites in higher mammals (Walkley et al., 2000). In addition, Jin et al. (Jin et al., 2003) did not observe extra dendrites in BDNF-treated interneurons of mouse cortex. However, the developmental time-course seems more important. It is possible that ferret neurons at the ages analyzed were prone to sprout extra dendrites, whereas rat neurons were already beyond the period of malleability of primary neurite patterning. Evidence comes from two recent studies. Danzer et al. (Danzer et al., 2002) reported extra dendrites after virally driven BDNF overexpression in hippocampal granule cells at the inner blade of the fascia dentata, not in outer parts or in CA pyramidal cells, suggesting that this structural plasticity can be evoked only in cells of a certain developmental stage. By contrast, Tolwani et al. (Tolwani et al., 2002) found all granule cells affected in mice overexpressing BDNF from actin promoter. The neurons have more dendrites with more branches rather than being longer. The promoter is active from embryonic time onwards overlapping with continuous granule cells neurogenesis, and newly generated neurons cannot avoid exposure to higher BDNF levels with the consequence that even primary neurite patterning becomes altered.

BDNF and NT4/5 accelerate dendritic differentiation of interneurons

Some hippocampal interneurons express NT3 or NGF (Pascual et al., 1998) and thus can process and release neurotrophins. BDNF is not in interneurons although they heavily express TrkB receptors (Rocamora et al., 1996; Gorba and Wahle, 1999) for consumption of BDNF-derived from pyramidal producers. However, pyramidal cell degeneration evokes BDNF expression in interneurons suggestive of an autocrine rescue response compensating the loss of target-derived factor (Wang et al., 1998). We now showed that BDNF and NT4/5 equipotently increased interneuronal complexity with less influence on dendritic elongation.

Concerning NT4/5, our results differ from recent results of Jin et al. (Jin et al., 2003), who found no effect of NT4/5 on the total dendritic length and the total number of dendritic branch points. The authors analyzed OTC prepared from mice,

which were about 5 days older than ours. Taking into account that the time course of development is shorter in mice, the age difference might be important. In line with our data we rather suggest that the neurons grow with NT4/5 at times when electrical activity is still low, but switch to BDNF as soon as activity increases.

Our results suggest that interneurons, when made into BDNF/NT4/5 producers, could use the factors for dendritic differentiation presumably in an autocrine fashion. An additional trans-synaptic (VonBartheld et al., 1996; Kohara et al., 2001) or paracrine action of TrkB ligands from pyramidal transfectants in the same culture could not be excluded, but appeared less likely (see above). In particular, intrinsic axonal connections of pyramidal cells are underdeveloped in an early stage as is the expression of synapse proteins (Kierstein et al., 1996). In vivo, adult synapse densities are reached around P20 (Blue and Parnavelas, 1983) and even adult pyramidal cell axons form only few synapses with a given multipolar interneuron (Buhl et al., 1997). Moreover, the multipolar neurons analyzed here mainly represent presumptive basket cells and their typical axosomatic contacts become recognizable during the third postnatal week, which largely rules out retrograde signaling in DIV 5-10 neurons. Furthermore, synaptic neurotrophin release requires activity, which is low in young OTC (Klostermann and Wahle, 1999). Together, this suggested TrkB ligands as potent autocrine mediators of interneuronal and pyramidal cell dendritogenesis.

Endogenous BDNF is not essential for pyramidal cell dendritogenesis

Owing to the complex interdependence of neuronal activity, afferents and in particular BDNF, activity deprivation studies delivered opposing results depending on developmental stage of the cell class, manner and timing of manipulation, and experimental conditions (see Introduction) (for a review, see McAllister, 2000). In our OTC, the lack of activity and BDNF did not compromise dendritic growth and complexity. However, the lack of endogenous NT4/5 retarded dendritogenesis suggesting that NT4/5 is more important than BDNF at least during the first two postnatal weeks. The surprisingly normal neocortical dendritogenesis without BDNF appeared to contradict the results from the overexpression study. Yet, the lack of BDNF was presumably compensated for by the action of NT4/5 and possibly also of NT3. In the absence of electrical activity the NT4/5 mRNA expression is upregulated. Previous studies also showed that NT4/5 and NT3 are activity-independently expressed (Gorba et al., 1999; Ichisaka et al., 2003).

Both factors are released via the constitutive secretory pathway in the absence of BDNF (for a review, see Lessmann et al., 2003). NT3 increases dendritic complexity of hippocampal and cortical pyramidal cells independent of glutamate-mediated transmission (Morfini et al., 1994; Baker et al., 1998), and NT4/5 promotes the dendritic development (this study) and the neuropeptide Y expression in the absence of activity (Wirth et al., 1998). Although apical dendrites are fairly promiscuous, NT4/5 and NT3 are highly effective (e.g. on layer VI basal dendrites), and NT3 actions even improve in the absence of TrkB ligands (McAllister et al., 1995; McAllister et al., 1997). Furthermore, layer II/III dendrites develop normally in dark-reared visual cortex (Tieman et al.,

1995) despite reduced expression of BDNF, but not of other neurotrophins (Schoups et al., 1995; Ichisaka et al., 2003), and reduced Trk receptor activation (Viegi et al., 2002).

The lower spine density in adult active OTC presumably reflected the fact that monocultures develop only the intrinsic wiring. Densities remained equivalent to the spine equipment displayed around P10 in vivo (Miller, 1981; Petit et al., 1988). Hippocampal OTC also have lower spine densities because OTC lack spine-targeting afferents and afferent drive known to promote spine development (Engert and Bonhoeffer, 1999; Toni et al., 1999; Knott et al., 2002). The higher spine densities in deprived OTC were surprising. However, Harris (Harris, 1999) proposes that lower excitation increases and higher excitation decreases spine density. Hippocampal slices instantly produce excessive spine densities owing to the sudden fall in activity (Kirov et al., 1999), as do activity-deprived Purkinje (Bravin et al., 1999) and thalamic neurons (Dalva et al., 1994; Rocha and Sur, 1995). Our neurons developed and maintained more spines for weeks under blockade and displayed higher synaptophysin expression (Kierstein et al., 1996). The lack of activity does not prevent circuit formation and indeed neurons resume synaptically evoked action potential activity upon recovery (Gorba et al., 1999). However, it might prevent activity-dependent pruning of spines and intrinsic connectivity.

Growing dendrites and shaping dendrites are thus two separate consecutive processes. Our OTC were prepared at birth and largely before cortical glutamatergic afferents and intrinsic connections become established. During these early stages, neurite growth appears activity independent. With time, activity-dependent control mechanism dominate to use-dependently shape cortical structure and function (Katz and Shatz, 1996), and activity-dependent BDNF starts to control speed and mode of dendritogenesis (Yacoubian and Lo, 2000). However, neurons that are activity deprived from birth never experience the activity-dependent mechanisms and instead continue to grow with high fidelity by activity-independent mechanisms. It is possible that young neurons, once they have switched to activity-dependent control, suffer under deprivation because they lose the competence to grow by activity-independent mechanisms.

The authors thank Dr George Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, USA) for the generous gift of the pCMX-hNT4myc expression plasmid, Dr Yves Alain Barde (MPI Martinsried, Germany) for the pCMV5-BDNF expression plasmid. This research was supported by DFG 'Neurovision' to P.W. and KOGNET graduate program to M.J.W.

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