Development/Plasticity/Repair

Brain-Derived Neurotrophic Factor Mediates Activity-Dependent Dendritic Growth in Nonpyramidal Neocortical Interneurons in Developing Organotypic Cultures

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Brain-derived neurotrophic factor (BDNF) promotes postnatal maturation of GABAergic inhibition in the cerebral and cerebellar cortices, and its expression and release are enhanced by neuronal activity, suggesting that it acts in a feedback manner to maintain a balance between excitation and inhibition during development. BDNF promotes differentiation of cerebellar, hippocampal, and neostriatal inhibitory neurons, but its effects on the dendritic development of neocortical inhibitory interneurons remain unknown. Here, we show that BDNF mediates depolarization-induced dendritic growth and branching in neocortical interneurons. To visualize inhibitory interneurons, we biolistically transfected organotypic cortical slice cultures from neonatal mice with green fluorescent protein (GFP) driven by the glutamic acid decarboxylase (GAD)67 promoter. Nearly all GAD67–GFP-expressing neurons were nonpyramidal, many contained GABA, and some expressed markers of neurochemically defined GABAergic subtypes, indicating that GAD67–GFP-expressing neurons were GABAergic. We traced dendritic trees from confocal images of the same GAD67–GFP-expressing neurons before and after a 5 d growth period, and quantified the change in total dendritic length (TDL) and total dendritic branch points (TDBPs) for each neuron. GAD67–GFP-expressing neurons growing in control medium exhibited a 20% increase in TDL, but in 200 ng/ml BDNF or 10 mM KCl, this increase nearly doubled and was accompanied by a significant increase in TDBPs. Blocking action potentials with TTX did not prevent the BDNF-induced growth, but antibodies against BDNF blocked the growth-promoting effect of KCl. We conclude that BDNF, released by neocortical pyramidal neurons in response to depolarization, enhances dendritic growth and branching in nearby inhibitory interneurons.

Key words: **biolistic; BDNF; GABA; GAD67; gene gun; inhibitory interneuron; neocortical development; neurotrophins; organotypic slice**

Introduction

GABAergic inhibition in the rodent cerebral cortex, although already functional at birth (Agmon et al., 1996; Wells et al., 2000), exhibits a protracted period of postnatal maturation lasting for several weeks (Komatsu and Iwakiri, 1991; Agmon and O'Dowd, 1992; Sutor and Luhmann, 1995; Cohen et al., 2000). Some of the developmental changes in inhibition occur postsynaptically, but others are presynaptic and include biochemical, morphological, and electrophysiological differentiation of GABAergic interneurons (Miller, 1986; Balcar et al., 1992; Micheva and Beaulieu, 1995; Guo et al., 1997; Massengill et al., 1997) and formation of GABAergic synapses (Micheva and Beaulieu, 1996; De Felipe et al., 1997; Marty et al., 2002). The delayed maturation of inhibition may allow increased NMDA receptor activation in the neonatal cortex, thereby opening a developmental window of enhanced plasticity during an early postnatal critical period (Agmon and O'Dowd, 1992; Kirkwood and Bear, 1994; Ramoa and McCormick, 1994; Rozas et al., 2001).

An attractive hypothesis that accounts for the delayed maturation of GABAergic inhibition suggests that inhibition is upregulated by neuronal excitation, thus establishing a negative feedback loop that counteracts the developmental increase in glutamatergic excitation, albeit with a lag (Marty et al., 1997; Bolton et al., 2000; Turrigiano and Nelson, 2000). A putative mediator of various effects of electrical activity is brain-derived neurotrophic factor (BDNF), a neurotrophin acting on tyrosine kinase (Trk)B receptors and implicated in neuronal development and synaptic plasticity (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999; Lu, 2003). In the cerebellum and hippocampus, activity-induced enhancement of GABAergic mechanisms can be mimicked by exogenous BDNF and/or prevented by BDNF inhibitors (Marty et al., 1996b, 2000; Rutherford et al., 1997; Seil and Drake-Baumann, 2000), suggesting that BDNF may also mediate activity-dependent maturation of inhibition. Consistent with this hypothesis, GABAergic maturation is accelerated in the cerebellum and cerebral cortex of BDNF-overexpressing mice

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(Bao et al., 1999; Huang et al., 1999; Aguado et al., 2003). Conversely, GABAergic maturation is retarded in the cerebellum and cerebral cortex of TrkB knock-out mice (Rico et al., 2002; Carmona et al., 2003) and in the cerebellum of the stargazer mouse, a mutant that is deficient in BDNF (Richardson and Leitch, 2002) [but see Olofsdotter et al. (2000) and Henneberger et al. (2002) for the apparently opposite result of enhanced inhibition in the dentate gyrus and superior colliculus of BDNF knock-out mice].

BDNF promotes neurochemical and dendritic differentiation of inhibitory neurons of the cerebellum, neostriatum, and hippocampus (Nawa et al., 1994; Ventimiglia et al., 1995; Marty et al., 1996a; Vicario-Abejon et al., 1998; Mertz et al., 2000), but its effects on dendritic growth of GABAergic interneurons in the neocortex remain unexplored. Here, we used a novel preparation (Jin et al., 2001) to follow the long-term dendritic development of individual GFP-expressing neocortical interneurons in organotypic cultures. We found that both BDNF and depolarization enhanced dendritic growth in nonpyramidal neocortical interneurons, and that the effect of depolarization was dependent on the release of endogenous BDNF.

Materials and Methods

Organotypic culture preparation. Organotypic cortical slice cultures were prepared as described previously (Stoppini et al., 1991; Jin et al., 2001). Postnatal day 1–3 mouse pups were anesthetized by cooling and decapitated, and their brains were rapidly removed and immersed in ice-cold artificial CSF (ACSF) (containing in mM: 126 NaCl, 3.0 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 26 $NaHCO_3$, and 20 dextrose, saturated with 95% O_2–5% CO_2). Under sterile conditions in a laminar flow hood, 350 μm-thick coronal brain slices of parietal cortex were cut with a Vibroslice (World Precision Instruments, Sarasota, FL). Slices were then placed on 30 mm Millicell membrane inserts (Millipore, Bedford, MA), usually two slices per insert, and the inserts were placed in individual wells of six-well plates containing 1 ml of culture medium. The culture medium was composed of the following (in ml per 100 ml): 46 Eagle's basal medium, 25 Earle's balanced salt solution, 25 horse serum, 1 mixture of penicillin (10,000 U/ml)–streptomycin (10 mg/ml)–glutamine (29.2 mg/ml) (all from Invitrogen, Carlsbad, CA), and 3 20% glucose solution. The slices were kept in a humidified incubator at 35°C with a 5% CO_2-enhanced atmosphere, and the medium was changed twice per week. After 2–3 d in culture, slices were transfected using a Helios gene gun (Bio-Rad, Hercules, CA).

Gene gun-mediated transfection. GAD67–GFP plasmid DNA was cloned from a 10.3 kb segment of the mouse GAD67 promoter region (Szabo et al., 1996; Katarova et al., 1998) fused in-frame to enhanced GFP (Clontech, Palo Alto, CA), as described previously (Jin et al., 2001). Cytomegalovirus (CMV)–GFP plasmid DNA (pGreen Lantern) was purchased from Invitrogen. Gene gun cartridges were prepared according to the manufacturer's protocol with slight modifications: for each 25 inch plastic tube, 12 mg of gold particles (1.0 or 1.6 μm diameter) and 25 μg of plasmid DNA (1 μg/μl in distilled water) were mixed in 100 μl of 50 mM spermidine, precipitated with 100 μl of 1 M $CaCl_2$, washed three times in 100% ethanol, and then resuspended in 3 ml of 0.02 mg/ml polyvinylpyrrolidone in ethanol and precipitated onto the internal wall of the plastic tube. Slices were bombarded with one cartridge per insert under 120 psi helium pressure through a nylon mesh that served to reduce the mechanical effects of the gas blast.

Confocal microscopy. Confocal images were first acquired at least 2 d after gene gun transfection to allow complete GFP filling of distal dendrites. Cultured brain slices were removed by cutting out the underlying Millicell membrane (leaving it adherent to the culture) and placed under sterile conditions in an imaging chamber filled with freshly oxygenated ACSF at room temperature. The imaging chamber was made of two round glass coverslips separated by 4 mm and held by a stainless-steel frame with a 17 mm-wide circular opening to allow imaging. Well separated neurons with bright GFP expression were selected for imaging. Neurons were imaged with a 20×, 0.7 numerical aperture (NA) dry objective on an inverted Zeiss (Thornwood, NY) LSM 510 laser-scanning confocal microscope, using the 488 nm argon laser line and a 505–550 nm bandpass emission filter. Image stacks (typically 10–20 optical sections per stack) were collected at 1.5–2.5 μm *z*-axis steps through the full extent of the dendritic tree and saved for off-line tracing and analysis. Images were taken with the lowest practical laser intensity and the shortest practical illumination time to limit photodynamic damage, and slices were kept outside the incubator for <1 hr per session. This imaging protocol was found not to cause any apparent ill effects to the imaged neurons or the slice culture, because most neurons remained viable and exhibited the same general dendritic morphology when imaged again 5 or 10 d later (see Fig. 3), and some remained viable even after repeated imaging over several weeks (data not shown). At the end of each imaging session, low-power (10×) images were taken in fluorescence and transmission modes to record the locations of the GFP-expressing neurons in the cortex relative to the pia and white matter. The slices with their adherent membranes were then placed back into multiwell plates under sterile conditions and returned to the incubator. The same neurons were imaged again 5 d later.

Pharmacological treatments. In some slices, one or more of the following were added to the culture medium: 200 ng/ml recombinant human BDNF (Alomone Labs, Jerusalem, Israel), 250 ng/ml recombinant human neurotrophin (NT)4/5 (courtesy of Genentech, South San Francisco, CA), 50 μg/ml rabbit anti-BDNF polyclonal antibody (Chemicon, Temecula, CA), 200 nM K252a, and 1 μM tetrodotoxin (TTX) (both from Alomone Labs). Treatments were refreshed on medium change. Pharmacological treatments started the day after the first imaging session; therefore, treatments lasted 4 of the 5 d between imaging sessions. Control experiments (slices cultured in normal medium) were interspersed throughout the treatment experiments.

Biocytin filling and histochemistry. To verify that GFP fluorescence revealed the full dendritic morphology, a subset of GFP-expressing neurons were filled with biocytin after confocal imaging. For biocytin filling, slices with the underlying Millicell membranes were transferred to a submersion recording chamber and continuously superfused with ACSF saturated with 95% O_2–5% CO_2 at room temperature. GFP-expressing neurons were visualized with a 40×, 0.8 NA water immersion objective under an Olympus Optical (Melville, NY) BX50 upright microscope equipped with fluorescence and differential interference contrast optics and a Hamamatsu (Bridgewater, NJ) CCD camera. Patch pipettes were pulled from 1.5 mm outer diameter glass capillary tubes (World Precision Instruments) and filled with a solution of 136 mM potassium gluconate, 2 mM $MgCl_2$, 0.6 mM EGTA, 10 mM HEPES, 2 mg/ml biocytin, with pH adjusted to 7.3 and osmolarity to 275–285 mOsm. A whole-cell clamp configuration was achieved using the Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and maintained for ~1 hr to allow for complete diffusion of biocytin. Slices were then fixed overnight in 0.1 M PBS with 4% paraformaldehyde at 4°C. After three rinses in PBS, fixed slices were incubated for 2 hr with ABC solution (Vector Laboratories, Burlingame, CA), followed by additional rinses in PBS and staining with diaminobenzidine (DAB) (0.7 mg/ml) and H_2O_2 (0.3%) in PBS. The reaction was stopped by transferring the slices to cold PBS.

Immunocytochemistry. For immunocytochemistry, GAD67–GFP-transfected slices were fixed for ~1 hr at room temperature and then overnight at 4°C in 4% paraformaldehyde in PBS, rinsed three times in PBS, and blocked for 1–3 hr in PBS containing 5–10% normal horse serum and 0.5–1% Triton X-100. Slices were then incubated for 2–3 d at 4°C with one or two of the following antibodies (at 1:1000 in PBS containing 1–5% normal horse serum and 0.1–0.5% Triton X-100): rabbit anti-GAD (AB108; Chemicon), mouse monoclonal anti-parvalbumin (P3088; Sigma, St. Louis, MO), mouse monoclonal anti-calbindin D28k (AB1778; Chemicon), rabbit polyclonal anti-calretinin (AB149; Chemicon), rabbit polyclonal anti-somatostatin (T-4103; Peninsula Laboratories, San Carlos, CA), rabbit polyclonal anti-neuropeptide Y (T-4070; Peninsula Laboratories), and rabbit polyclonal anti-GABA [gift of David Pow (University of Queensland, St. Lucia, Australia)] [this antibody was designed for paraformaldehyde-only fixation, and its specificity was established previously (Pow et al., 1995; Spirou and Berrebi, 1997)]. After incubation with antibody, slices were washed three times in PBS, fol-

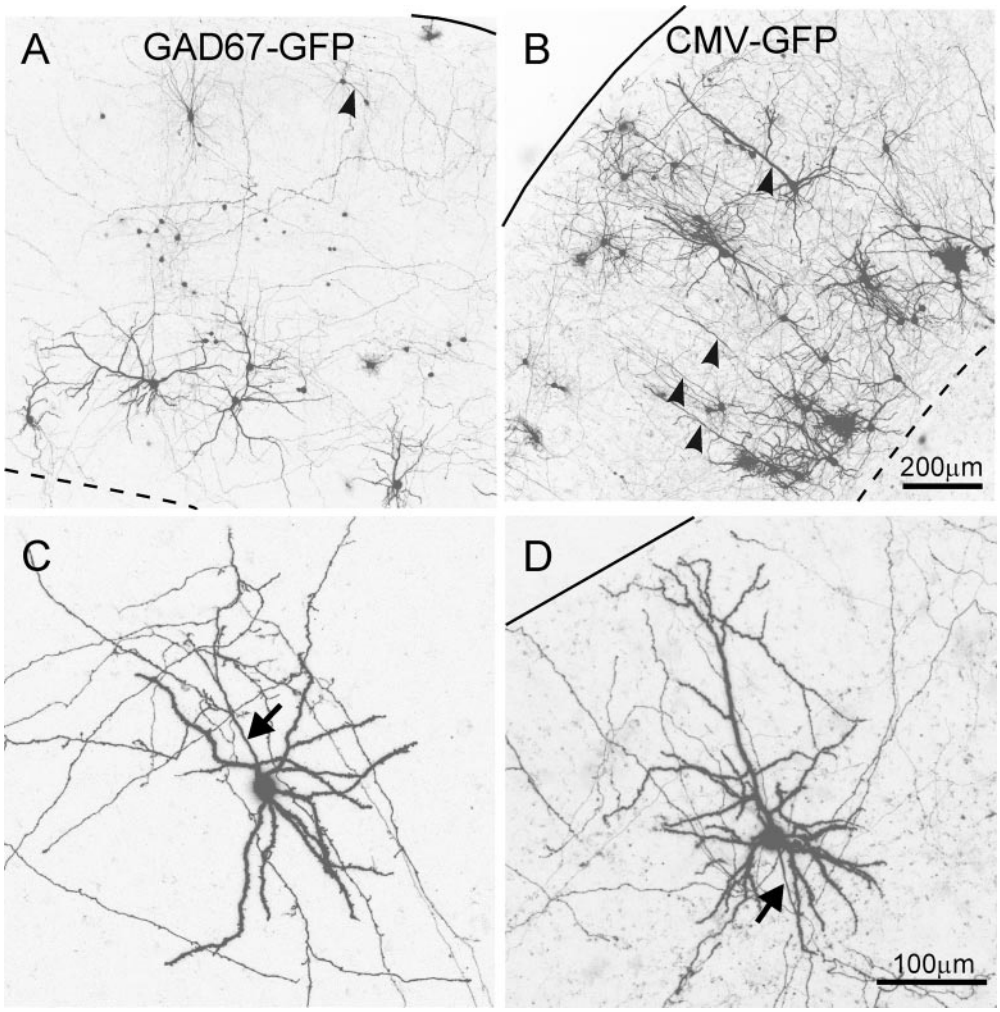

Figure 1. Neurons expressing GAD67–GFP were nonpyramidal, but neurons expressing CMV–GFP had mixed morphologies. *A*, *C*, Neurons labeled by transfection with GAD67–GFP, imaged at low (10×) and high (20×) power, respectively. *B*, *D*, Neurons labeled by transfection with CMV–GFP, imaged at low and high power, respectively. The images in *A–D* are from four different slices. Arrowhead in *A* points to a pair of apparently fused neurons; arrowheads in *B* indicate apical dendrites of pyramidal neurons. Arrows in *C* and *D* indicate the initial segment of the axon, which exits toward the pial surface in the interneuron (*C*) and toward the white matter in the pyramidal neuron (*D*). Solid lines in this and subsequent figures indicate the pial surface, and dashed lines indicate the border between layer 6 and the white matter. Scale bars: (in *B*) *A*, *B*, 200 μm; (in *D*) *C*, *D*, 100 μm.

lowed by one or both of the following secondary antibodies (at 1:1000 in PBS with 1% normal horse serum): Alexa 546 goat anti-rabbit IgG $(ab')_2$ fragments or Alexa 633 goat anti-mouse IgG $(ab')_2$ fragments (both from Molecular Probes, Eugene, OR), for 2 hr at room temperature. The slices were then washed three times in PBS, mounted in Vectashield (Vector Laboratories), and coverslipped.

Neuronal tracing. Neurons with strong GFP fluorescence throughout their dendritic tree were reconstructed digitally from the confocal image stacks using Neurolucida software (MicroBrightField, Colchester, VT). GFP-expressing neurons that were filled with biocytin were traced a second time with Neurolucida after processing the DAB reaction, using a 20×, 0.7 NA objective and a CCD video camera attached to an Olympus Optical AX70 microscope.

Data analysis. Quantitative analysis on the traced data were done using Neuroexplorer software (MicroBrightField). Numerical data (total dendritic length and branch points) generated from the second imaging session (day 5) were divided by the values derived from the first imaging session (day 0) to yield a ratio; geometrical means and SEs of the ratios were then calculated for each treatment group and for the control group.

Statistics. Exact, distribution-independent permutation tests were used to compare experimental treatments with control (Manly, 1997; Good, 1999). Computations were done using MathCad (MathSoft, Cambridge, MA). When a group mean was compared with an expected value, the binomial sign test (Fisher's one-sample randomization test) was used: each data point was assigned a positive or a negative sign on the basis of its value relative to the expected mean, and the probability that an equal or more extreme distribution of signs would occur at random was calculated from the binomial probability function. When the means of two groups were compared, the difference between the means was calculated for 10,000 random permutations of the data, and the fraction of values equal to or more extreme than the experimental observation was doubled to yield a two-tailed *p* value. All of the comparisons were made pairwise, in general between each experimental group and the control group, but in some cases (indicated) between two experimental groups, with no adjustments for multiple comparisons (Rothman, 1990; Savitz and Olshan, 1998).

Results

Transfecting GABAergic interneurons with GAD67–GFP

To visualize nonpyramidal neocortical interneurons, we transfected cortical organotypic slices with a DNA construct in which the GAD67 promoter drives the expression of GFP (Jin et al., 2001), because GAD is a high-fidelity marker for GABAergic neurons (Mugnaini and Oertel, 1985). Two days after transfection, up to a few dozen neurons per slice expressed GFP fluorescence at varying levels of intensity (Fig. 1*A*). In strongly fluorescent neurons, minute morphological details were discernible, including

dendritic filopodia and spines and fine axonal arborizations (Fig. 1*C*). GABAergic neurons in the cerebral cortex exhibit a nonpyramidal morphology (Houser et al., 1983; Meinecke and Peters, 1987; Prieto et al., 1994); therefore, to confirm that GAD67–GFP expression is restricted to GABAergic interneurons, we compared the morphology of GAD67–GFP-expressing neurons with that of neurons transfected with a GFP construct driven by the nonspecific CMV promoter (CMV–GFP) (Fig. 1*B*). Neocortical neurons with a long vertical dendrite extending toward the pia and several additional primary dendrites growing in all of the other directions were defined as pyramidal-like (Fig. 1*D*), and all of the other neurons were defined as nonpyramidal. In GAD67–GFP-transfected slices, 92% of GFP-expressing neurons were nonpyramidal ($n = 212$ neurons) (Fig. 1*A*,*C*); in contrast, CMV—GFP-expressing neurons were divided about evenly between pyramidal (45%) and nonpyramidal (55%) morphologies ($n = 71$) (Fig. 1*B*,*D*). The difference between slices transfected with GAD67–GFP and CMV–GFP was highly significant ($p < 10^{-10}$; Fisher's exact test). The higher percentage of nonpyramidal neurons in CMV–GFP-transfected slices compared with the known incidence of GABAergic neurons in the rodent neocortex (15–25%) (Ren et al., 1992; Micheva and Beaulieu, 1995) could have reflected a higher efficacy of the CMV promoter in GABAergic neurons, although misclassification of some pyramidal neurons as nonpyramidal because of truncation of their apical dendrites during the slicing procedure, or CMV–GFP expression by spiny stellate cells (layer 4 neurons that are glutamatergic but nonpyramidal), cannot be ruled out. Nevertheless, the small fraction (8%) of neurons with pyramidal-like morphology among hundreds of GAD67–GFP-expressing cells strongly suggests that GAD67–GFP expression was primarily restricted to GABAergic interneurons. GAD67–GFP-expressing cells with pyramidal-like morphology were excluded from additional study.

In many slices, a small number of GFP-expressing neurons were observed to form clusters of two to three closely situated cells that appeared to be fused together at a single contact point on their somata or their proximal dendrites (Fig. 1*A*, arrowhead). Because such fused neurons were found in both GAD67–GFP- and CMV–GFP-transfected slices, we assume that the fusion was induced by the mechanical effect of the biolistic transfection; however, the precise mechanism remains to be determined. Fused neurons were excluded from additional study.

As an additional test of the neurotransmitter content of GAD67–GFP-expressing neurons, we stained them immunocytochemically with antibodies to GABA (Fig. 2*A*,*B*) and to GAD67 (Fig. 2*C*,*D*). Forty-two percent of GAD67–GFP-expressing neurons examined were immunopositive for GABA ($n = 24$), but only 26% were immunopositive for GAD67 ($n = 34$). Moreover, GAD67–GFP-expressing neurons staining immunopositive for either GABA or GAD67 were among the least intensely stained neurons in their microscopic field (Fig. 2*B*,*D*). This could reflect a low level of GAD67 (and therefore GABA) expression in GFP-expressing neurons, possibly because of competition for limited transcription factors between the endogenous and exogenous GAD67 promoters.

Cortical GABAergic interneurons can be classified into several different subtypes with distinct neurochemical identities (Kubota et al., 1994; Kawaguchi and Kubota, 1997). To test whether biolistic transfection with GAD67–GFP preferentially labeled GABAergic interneurons from one or more of these classes, we immunostained GAD67–GFP-transfected slices for the calcium-binding proteins parvalbumin (PV), calbindin (CB), and calretinin (CR) and for the neuropeptides somatostatin (SOM) and neuropeptide Y (NPY). In this analysis, we also included GAD67–GFP-expressing neurons in the hippocampus, because the neurochemical composition of the hippocampus is virtually identical to that of the neocortex, and because many of the GABAergic interneuron classes in the hippocampus can be readily recognized on the basis of their laminar position and morphological features (Freund and Buzsaki, 1996). Between 4 and 12% of GAD67–GFP-expressing neurons coexpressed any one of these markers (PV, 7.4% of 189 cells; CB, 6.7% of 74 cells; CR, 4% of 51 cells; SOM, 12.1% of 157 cells; NPY, 4% of 25 cells).

The incidence of GABAergic subtype markers in GAD67–GFP-expressing neurons was well below the incidence of the same markers in the GABAergic population of the mature cortex *in vivo*; for example, approximately one-half of all of the GABAergic neurons are estimated to express PV in the adult rat neocortex (Ren et al., 1992). Our immunostaining experiments, however, were done on slices younger than 2 weeks of equivalent age (we define equivalent age as postnatal day at culturing plus days *in vitro*), when PV mRNA and protein are just becoming detectable *in vivo* (Alcantara et al., 1993; del Rio et al., 1994; de Lecea et al., 1995). Moreover, PV expression is considerably reduced in neocortical slice cultures prepared from neonatal animals, even after several weeks in culture (Vogt Weisenhorn et al., 1998).

Unlike parvalbumin, globally reduced expression could not underlie the low incidence of somatostatin in GAD67–GFP-expressing cells, as demonstrated by two contrasting examples in Figure 2*E–H*. The large GFP-expressing neuron in Figure 2*E*, with a cell body at the oriens–alveus border of CA1, was by its laminar location and dendritic morphology a typical oriens–lacunosum moleculare interneuron, a class of hippocampal interneurons known to express SOM (Freund and Buzsaki, 1996), and was indeed SOM immunopositive (Fig. 2*F*). In contrast, the GFP-expressing neuron in Figure 2*G*, with its cell body in cortical layer 6, was by its ascending axons (inset) an unmistakable Martinotti cell, a class of cortical interneurons also known to contain SOM (Wahle, 1993; Kawaguchi and Kubota, 1996); however, this cell was SOM immunonegative (Fig. 2*H*), although several non-GFP-expressing neurons in the same microscopic field were clearly immunopositive. These examples show that the low incidence of somatostatin in GAD67–GFP-expressing neurons did not result from a general low level of immunoreactivity to this peptide, nor could it be attributable to an inadvertent inclusion in our sample of glutamatergic neurons. Most likely, it reflected lower levels of SOM in GAD67–GFP-expressing neurons belonging to this GABAergic subtype, possibly because of diversion of their biochemical resources to the production of large amounts of an exogenous protein (GFP). It should be noted that, in mice carrying a GAD67–GFP transgene, neurons coexpressing GFP and SOM are often less intensely stained (for SOM) than non-GFP-expressing, SOM-immunopositive neurons nearby (our unpublished observations), indicating similar competitive pressures in transgenic animals, although the transgene is presumably present in the genome of transgenic mice in far fewer copies than those of the GAD67–GFP construct in biolistically transfected neurons.

Dendritic development of nonpyramidal cortical interneurons in organotypic slice cultures

Because GFP expression in our cultures revealed the detailed morphology of living neurons, we were able to follow the morphological development of individual neurons over time by imaging the same neuron two or more times at 5 d intervals, digitally

reconstructing the three-dimensional (3-D) dendritic morphology of the neuron from stacks of confocal images, and quantifying the TDL and TDBPs at each imaging time point.

We first followed the growth of GFP-expressing neurons in normal medium (Fig. 3). When first imaged, the age of our cultures varied between equivalent postnatal day 7 (EP7) (equivalent postnatal day = postnatal day at culturing + days *in vitro*) and EP24. We quantified dendritic growth over the next 5 d by calculating the ratio between TDL at second imaging (day 5) and TDL at first imaging (day 0). This ratio is plotted in Figure 3*C* against equivalent age at first imaging. The mean TDL ratio was 1.22 ± 0.07 ($n = 19$ neurons) for neurons first imaged at EP10 or earlier (left of vertical dotted line), but was 1.01 ± 0.03 ($n = 26$) for neurons first imaged at older ages (right of dotted line). The difference between the means of the two groups was highly significant ($p = 0.0008$). Notably, the SD of the TDL ratios in the younger group was twice as large as in the older group, indicating a higher variability in growth rate in younger slices. As an additional measure of growth, we also compared the TDBP ratio of the second to the first imaging. The mean TDBP ratio (0.93 ± 0.09 and 1.03 ± 0.06, respectively, for the younger and older groups) was not significantly different from 1 for either group ($p = 0.5$ and 0.18, respectively), indicating that, in our control conditions, there was a balance between the addition and elimination of new branches, even when (as in the younger age group) there was a net elongation of dendrites. These data suggest that dendritic growth in our nonpyramidal cortical interneurons occurred mostly during the first 2 weeks in organotypic culture, and that dendritic branching patterns may have been already fully established by the age of our earliest imaging (EP7). Because our goal was to study the role of BDNF during normal development, all of the subsequent experiments were done on the younger age group (EP7–EP10 at first imaging).

Because organotypic slices grow thinner and more translucent with time in culture, and because the level of GFP expression often appeared to increase with time in culture, we were concerned that any apparent increase in dendritic length between the two imaging sessions could be the result of incomplete visualization or incomplete GFP filling of dendrites at the earliest imaging time point. To test this possibility, we compared dendritic filling and visualization between GFP and biocy-

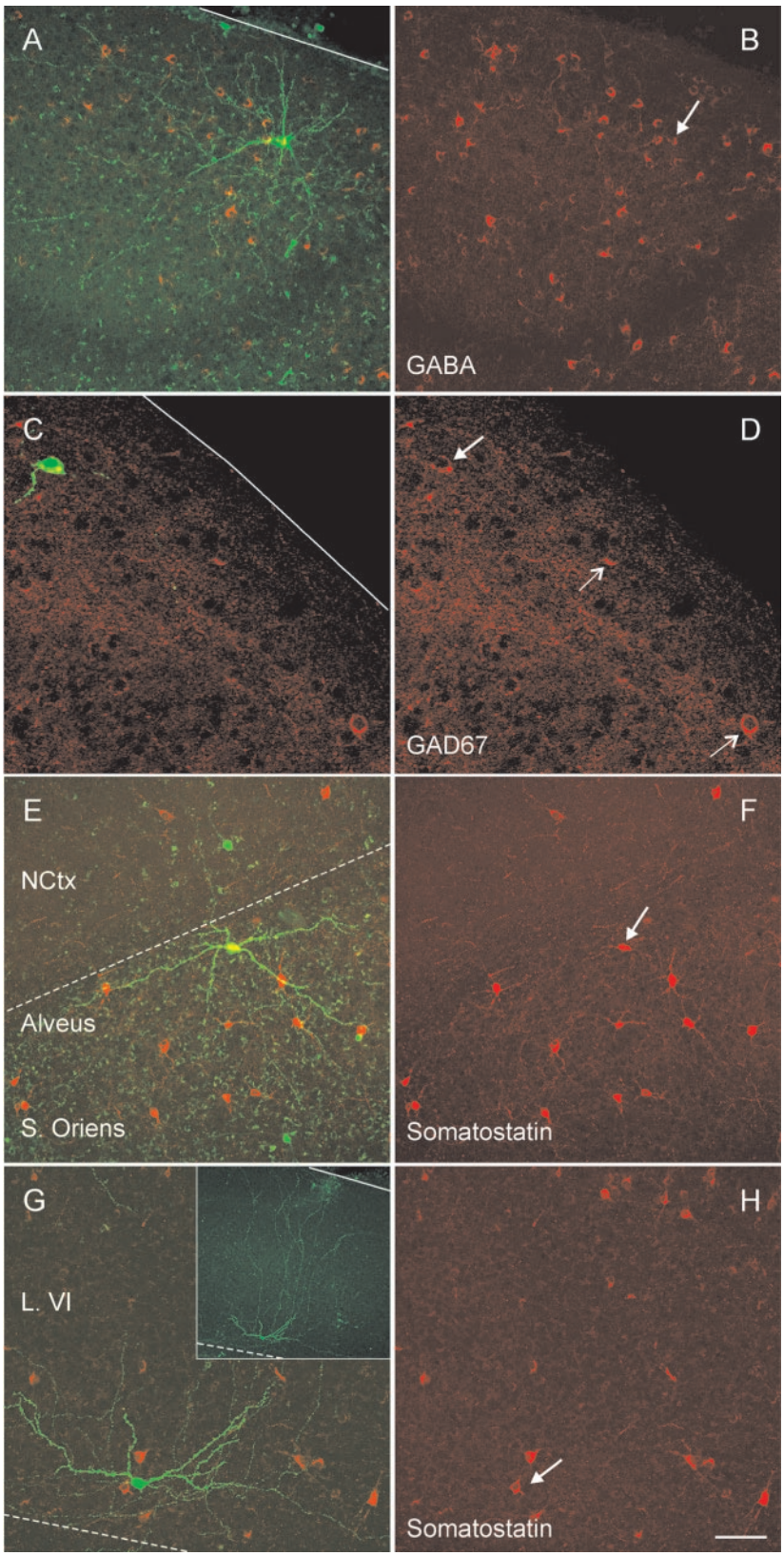

Figure 2. Some, but not all, of the GAD67–GFP-expressing neurons were immunopositive for GABA and GABAergic subtype markers. *A*, *C*, *E*, *G*, Dual-channel confocal images of immunofluorescence (pseudocolored red) and GAD67–GFP fluorescence (pseudocolored green). *B*, *D*, *F*, *H*, The same fields with immunofluorescence only. Immunostaining is for antibodies against GABA (*A*, *B*), GAD67 (*C*, *D*), and somatostatin (*E–H*). Arrows in *B*, *D*, *F*, and *H* indicate the location of the GFP-expressing neuron shown in *A*, *C*, *E*, and *G*, respectively. Note that the GFP-expressing neurons in *A*, *C*, and *E* were immunopositive, but the neuron in *G* was immunonegative. Light arrows in *D* indicate non-GFP-expressing immunopositive neurons. Scale bar: (in *H*) *A*, *B*, *E–H*, 100 μm; *C*, *D*, 40 μm. NCtx, Neocortex; S Oriens, stratum oriens; L. VI, layer 6. Solid and dashed lines are as described in Figure 1.

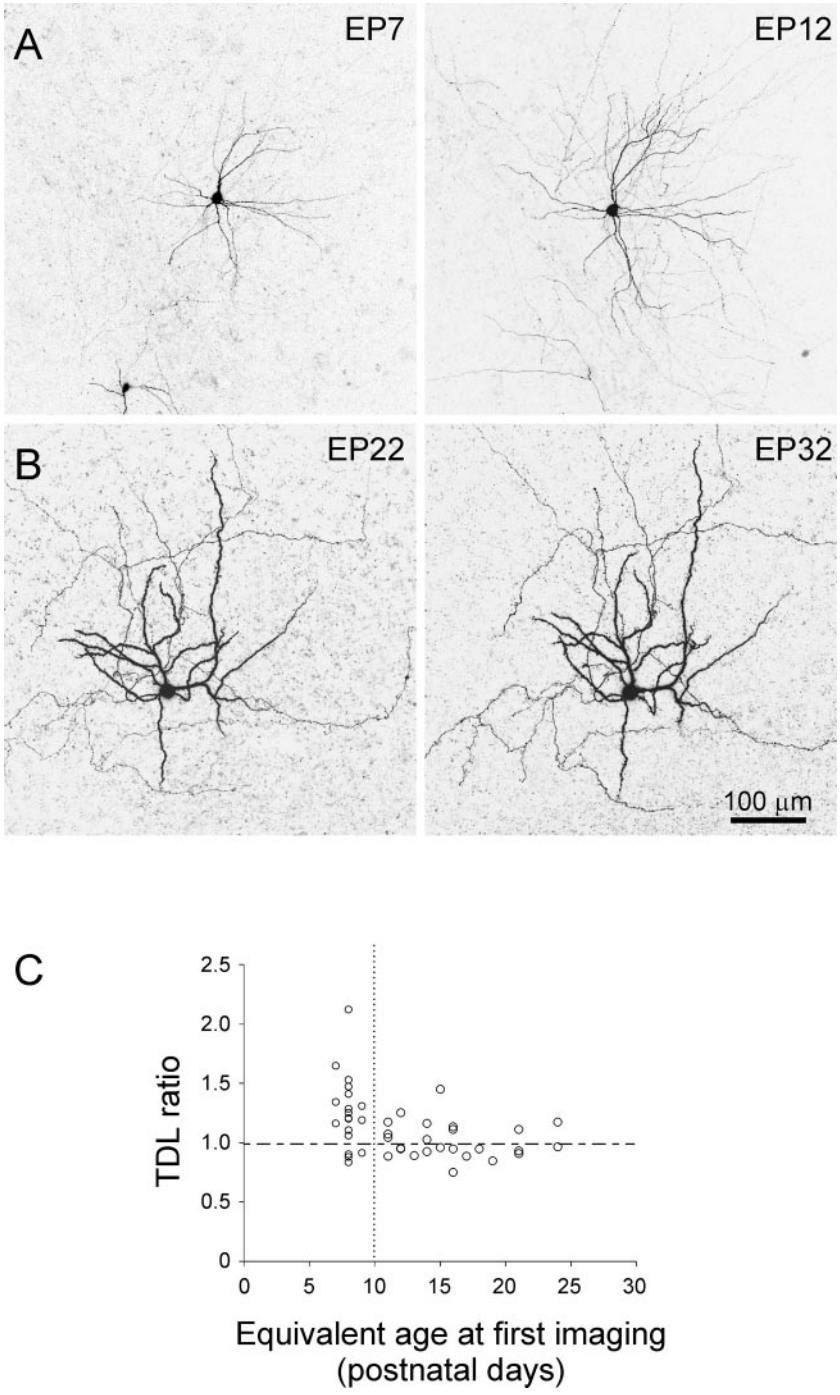

Figure 3. Dendritic growth of GAD67–GFP-expressing neurons in control medium occurred only in younger cultures. *A*, Confocal projection of a representative GAD67–GFP-expressing neuron first imaged at EP7 (left) and again 5 d later (right). *B*, Another neuron first imaged at EP22 (left) and again 10 d later (right). Scale bar: (in *B*) *A*, *B*, 100 μm. *C*, TDL growth ratio of all of the neurons in our sample during a 5 d period in normal medium, plotted against the equivalent age at first imaging. A TDL ratio of 1 (dashed line) indicates no change. Note that dendritic growth was observed in neurons first imaged at EP7–EP9 (left of the vertical dotted line), but there was no change on average in neurons first imaged at later ages (right of the vertical dotted line).

tin, a much smaller molecule commonly used for reconstruction of neuronal morphology after intracellular recordings. Seven GFP-expressing neurons were imaged with a confocal microscope and then injected with biocytin through a patch pipette, processed for the histochemical DAB reaction, and traced with a computerized system. The 3-D dendritic morphology was then compared between reconstructed confocal images and biocytin tracings of the same neuron (Fig. 4). We found that, in older neurons, dendritic reconstructions from GFP and biocytin closely overlapped, after adjusting for shrinkage (Fig. 4*A*). In some of the younger neurons, however, GFP tracings from confocal stacks missed a terminal portion of one or two dendritic branches, as evident from comparison with the biocytin image (Fig. 4*B*, top tracing, arrow). This was not caused by insufficient GFP filling, but rather by loss of optical signal from dendritic branches growing deep into the tissue, as illustrated by the *x–z* projection in Figure 4*B*, bottom tracing, arrow. This situation was easy to identify, because such branches gradually disappeared from view in deeper optical sections; these branches were thereby excluded from analysis in all of the images taken from that particular neuron.

BDNF, but not NT4/5, enhanced dendritic growth in nonpyramidal interneurons

To investigate the role of BDNF and activity in regulating dendritic growth of nonpyramidal interneurons, we followed the morphological development of individual GAD67–GFP-expressing neurons over a 5 d period and compared TDL and TDBP ratios between neurons from cultures treated with 200 ng/ml BDNF, or one of several other treatments, to neurons from untreated control cultures. Representative neurons are illustrated in Figure 5, and a summary plot of all of the experiments is presented in Figure 6. As illustrated in Figure 5*A*, growth in control conditions was modest (note the small rightward shift in the cumulative histogram). As reported above, the mean control TDL ratio (i.e., TDL at day 5 divided by TDL at day 0) was 1.22 ± 0.07 ($n = 19$); this change was significantly different from 1 ($p = 0.01$), but there was no significant change in TDBPs. In contrast, GAD67–GFP-expressing neurons developing in the presence of BDNF (Fig. 5*B*) exhibited considerably more growth. The mean TDL ratio in the presence of BDNF was 1.42 ± 0.05 ($n = 27$), representing a fractional increase (42%) nearly double that in control medium (22%). BDNF also promoted branching of existing dendrites (but not addition of primary dendrites): the mean TDBP ratio in BDNF was 1.25 ± 0.09. Both TDL and TDBP ratios in the BDNF group were significantly different from those of the control group ($p = 0.03$ and 0.01, respectively) (Fig. 6*A*,*B*). These results indicate that BDNF promotes dendritic growth and complexity in nonpyramidal neocortical interneurons.

To test whether the growth-promoting effect of BDNF on nonpyramidal, GABAergic interneurons depended on electrical activity, we allowed cultures to develop in the presence of both 200 ng/ml

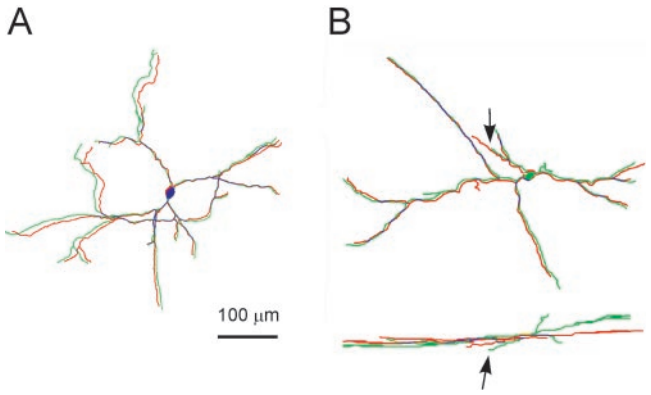

Figure 4. GAD67–GFP fluorescence revealed the full dendritic morphology. GAD67–GFP-expressing neurons were imaged 3 weeks (*A*) or 3 d (*B*) after transfection and then injected with biocytin through a patch pipette. The 3-D reconstructions of the dendritic trees from the confocal images (green) are superimposed on the 3-D reconstructions from the biocytin tracings of the same neurons (red). Note that the GFP fluorescence was at least as extensive as the biocytin labeling, but one secondary dendrite in the younger neuron in *B* appeared shorter in the confocal image compared with the corresponding biocytin tracing (*B*, top tracing, arrow). The terminal part of the dendrite was missing from the confocal reconstruction, because it was located deep in the slice, as shown by the *x–z* image of the same neuron (*B*, bottom tracing, arrow). Scale bar, 100 μm (for both images).

BDNF and 1 μM TTX (Fig. 5*C*). Under these conditions, there was still enhanced dendritic growth in GAD67–GFP-expressing neurons, and the mean TDL ratio was 1.62 ± 0.11 ($n = 17$), significantly larger than control ($p = 0.001$) (Fig. 6*A*) (the increase in TDL ratio compared with BDNF alone was only marginally significant at the $p = 0.06$ level). TTX did seem to prevent branching (Fig. 6*B*) ($p = 0.6$). These data suggest that, unlike the effect of BDNF on pyramidal neurons (McAllister et al., 1996), the enhancing effect of BDNF on dendritic growth of GABAergic interneurons does not require sodium-dependent electrical activity.

To study the role of endogenous BDNF in dendritic development in culture, we compared dendritic growth in control slices with growth in cultures in which endogenous BDNF was neutralized with a high titer (50 μg/ml) of anti-BDNF antibodies, shown to block the effect of endogenous BDNF (Ghosh et al., 1994; Marty, 2000; Seil and Drake-Baumann, 2000), or in which Trk signaling was blocked by 200 nM Trk signaling inhibitor K252a (Knusel and Hefti, 1992). Under both conditions, dendritic growth was significantly reduced compared with control (Fig. 6*A*) (TDL ratios were 1.07 ± 0.04, $n = 21$, $p = 0.03$ for anti-BDNF; 0.99 ± 0.05, $n = 14$, $p = 0.006$ for K252a), indicating that dendritic growth *in vitro* is regulated by release of endogenous BDNF into the extracellular milieu. Finally, we tested the effect of the neurotrophin NT4/5, which also activates TrkB receptors and is expressed in the cortex during early development (Maisonpierre et al., 1990; Timmusk et al., 1993). NT4/5 (250 ng/ml) had no enhancing effect on dendritic growth or branching of nonpyramidal interneurons; indeed, growth in NT4/5 was significantly reduced (Fig. 6*A*) (1.07 ± 0.05, $n = 14$, $p = 0.03$), possibly because NT4/5 competed with endogenous BDNF for TrkB binding sites (Janiga et al., 2000).

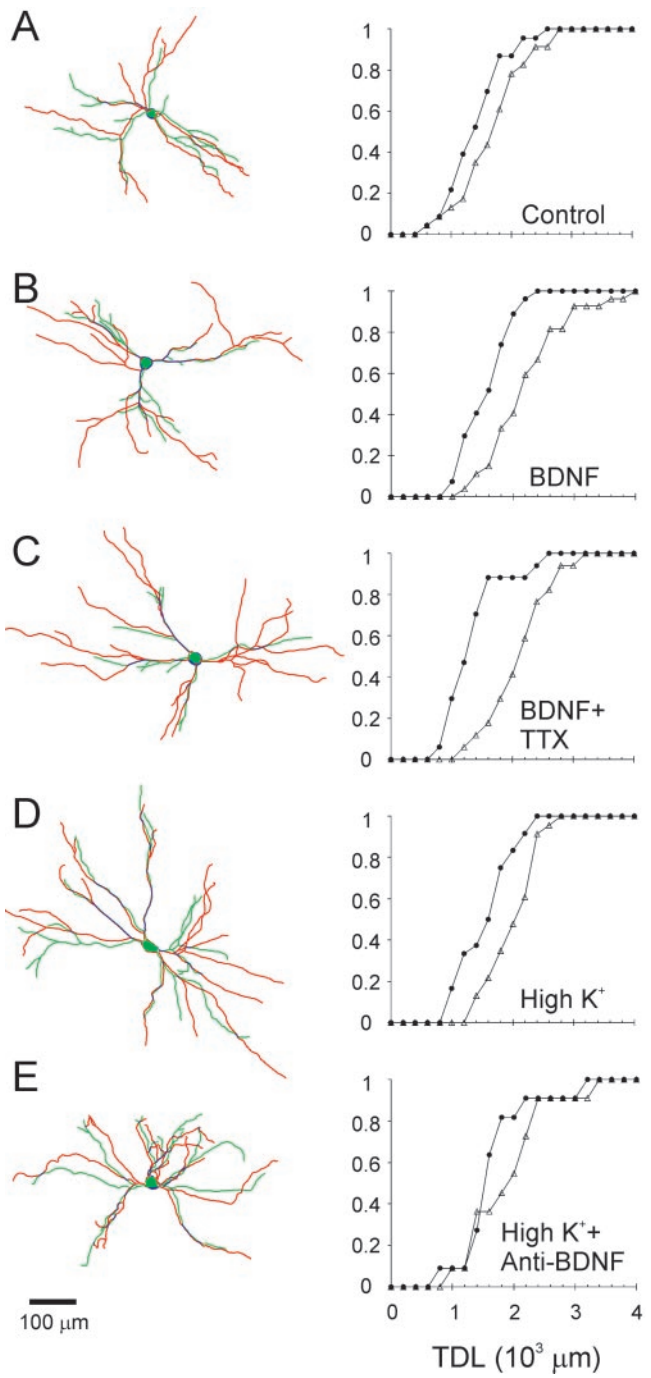

Figure 5. BDNF and KCl enhanced dendritic growth of GAD67–GFP-expressing neurons. Left column, Morphological reconstructions of representative dendritic trees of neurons before (green) and after (red) a 5 d period in culture medium supplemented by one of the following: no supplement (*A*), 200 ng/ml BDNF (*B*), 200 ng/ml BDNF and 1 μM TTX (*C*), 10 mM KCl (*D*), or 10 mM KCl and 50 μg/ml anti-BDNF (*E*). Right column, Cumulative histogram of TDL in each treatment group; each data point represents the fraction of neurons in the treatment group with TDL values equal to or smaller than the corresponding *x* value. ●, TDL at day 0 (before treatment). △, TDL at day 5 (after treatment). Note that the rightward shift of the curves (indicating an overall increase in total dendritic length) was considerably larger in *B*, *C*, and *D*, compared with control. Scale bar, 100 μm (for all images).

Effect of neuronal activity on GABAergic neuron dendritic growth

Electrical activity can increase several parameters of GABAergic function (Rutherford et al., 1997; Marty et al., 2000), but its effect on dendritic growth of cortical GABAergic neurons was not known. We tested the effect of electrical activity on GAD67–GFP-expressing neurons by incubating slice cultures in a medium containing 10 mM KCl (Fig. 5*D*), which depolarizes cortical neurons and causes action potential firing (Franklin et al., 1995; Tongiorgi et al., 1997; Vaillant et al., 2002). GAD67–GFP neurons cultured in a high KCl medium exhibited enhanced growth and branching

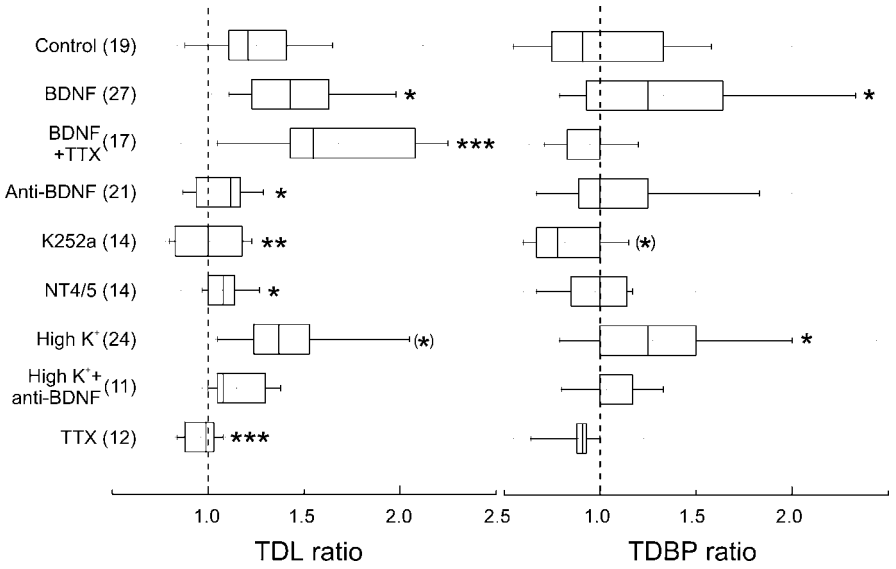

Figure 6. Summary and statistical analysis of the effects of all of the tested treatments on dendritic growth and branching. Ratio change in TDL (left) and TDBPs (right) after 5 d of the treatment indicated. Each box spans the 25th to 75th percentile of the data points, with the median represented by a vertical line inside the box; the whiskers span the 5th to 95th percentiles. The number of neurons tested is indicated in parentheses after the treatment label. The dashed vertical lines at $x = 1$ indicate no change. $^{(*)}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$; p values are from pairwise comparisons with the control group.

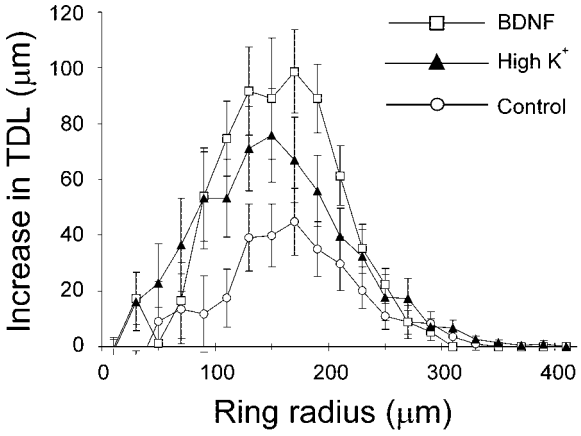

Figure 7. Sholl analysis reveals a similar pattern of dendritic growth in response to BDNF and KCl. Absolute increase in TDL in the control (○), high K^+ (▲), and BDNF (□) groups was calculated separately for each consecutive, 20 μm-wide concentric ring, and averaged between neurons. Note that the high K^+ and BDNF groups exhibited a greater average increase in TDL, compared with control, but the radial extent of the increase was similar among all three groups. Error bars represent SEMs.

compared with control (the mean TDL and TDBP ratios were 1.39 ± 0.06 and 1.22 ± 0.09, respectively; $n = 24$). The effect on branch points was statistically significant ($p = 0.03$); however, the effect on dendritic length was only marginally so ($p = 0.08$) (Fig. 6), most likely reflecting the modest level of depolarization expected from 10 mM KCl.

Because electrical activity enhances cortical BDNF levels (Castren et al., 1992; Tongiorgi et al., 1997), the effect of high K^+ could have been mediated through activity-dependent release of BDNF. Consistent with this interpretation, the effects of high KCl on dendritic growth and complexity were not significantly different from the effects of BDNF ($p = 0.76$ and 0.63, respectively). We tested the dependence of the KCl effect on released BDNF by neutralizing endogenous extracellular BDNF with anti-BDNF antibodies (Fig. 5*E*). Dendritic development in a medium supplemented with 10 mM KCl and 50 μg/ml anti-BDNF was nearly blocked: the TDL ratio was 1.14 ± 0.05, and the TDBP ratio was 1.02 ± 0.06 ($n = 11$). TDL in high KCl plus anti-BDNF was very significantly different from growth in KCl alone ($p = 0.002$), and TDBP was marginally so ($p = 0.07$), but both were not significantly different from growth and branching in anti-BDNF alone ($p = 0.3$ and 0.73, respectively), consistent with the interpretation that the dendritic growth-promoting effect of high KCl was indeed mediated by BDNF, rather than through an independent, additive pathway (because if the latter was the case, high KCl should have promoted some growth even in the presence of anti-BDNF).

To test whether growth in normal culture medium is dependent on electrical activity, we cultured slices in the presence of 1 μM TTX. Mean TDL ratio in TTX was 0.96 ± 0.03 ($n = 12$), very significantly different from control ($p = 0.0002$) (Fig. 6*A*), indicating that TTX counteracted the increase in TDL that occurred in normal medium and suggesting that sodium-dependent electrical activity is required for normal dendritic growth in culture.

Finally, to test whether BDNF and depolarization affect dendritic growth in a similar manner, we used Sholl analysis to quantify dendritic growth within consecutive concentric rings around the cell body, and compared Sholl plots showing the increase in dendritic length over a 5 d growth period among control, BDNF, and high K^+ conditions (Fig. 7). As illustrated, dendritic growth was distributed within a radius of ~300 μm around the cell body, with the most growth occurring between 50 and 250 μm from the cell body. No major differences were evident between growth patterns in BDNF and high K^+, consistent with the conclusion that growth under both conditions was mediated by the same cellular mechanisms.

Discussion

In this study, we tested whether exogenous BDNF or depolarization by high KCl enhanced dendritic growth of nonpyramidal cortical interneurons expressing GAD67–GFP in organotypic slice cultures, and whether there was cross-dependency between the effects of BDNF and depolarization. Short-term (minutes to hours) changes in dendritic morphology were tracked previously in living GFP-expressing cortical neurons *in vitro* (Fischer et al., 1998; Engert and Bonhoeffer, 1999; Horch et al., 1999; Maletic-Savatic et al., 1999; Wu et al., 2001) or *in vivo* (Lendvai et al., 2000), but our study is the first, to our knowledge, to follow the morphological development of the same neocortical neurons over 5 or more days. By imaging the same GFP-expressing neurons at 5 d intervals, we demonstrated that (1) both BDNF and elevated K^+ enhanced dendritic growth of nonpyramidal interneurons to the same degree, (2) the effect of BDNF was not dependent on neuronal activity, but (3) the KCl-induced dendritic growth was dependent on release of endogenous BDNF. Together, these observations suggest a role for BDNF in regulating structural and functional maturation of GABAergic interneurons in the developing neocortex and potentially mediating activity-induced dendritic remodeling in the adult.

BDNF effects in pyramidal and nonpyramidal neocortical interneurons are qualitatively different

Exogenous or endogenous BDNF, NT3, and NT4/5 have been shown to promote dendritic elongation and branching in neocortical pyramidal cells developing in organotypic cultures (McAllister et al., 1995, 1997; Horch et al., 1999; Niblock et al., 2000; Yacoubian and Lo, 2000), but their effects on nonpyramidal, inhibitory neocortical interneurons were not studied previously. Our results show that activation of TrkB receptors by BDNF, but not by NT4/5, promotes dendritic elongation and branching in developing neocortical nonpyramidal interneurons. Although both BDNF and NT4/5 activate TrkB receptors, the two ligands often affect dendritic growth differentially, even in the same neurons (McAllister et al., 1995; Bosco and Linden, 1999; Steljes et al., 1999).

In our study, a 4 d treatment with 200 ng/ml BDNF caused a 40% increase in TDL of neocortical interneurons. In contrast, a 36 hr treatment with 200 ng/ml BDNF doubles the TDL and TDBPs of layer 4 pyramidal neurons in the ferret (McAllister et al., 1995). We noted several other differences between the published effects of BDNF on neocortical pyramidal neurons and the effects on nonpyramidal neurons in this study: BDNF did not increase the number of primary dendrites in nonpyramidal neurons, but does so in pyramidal cells (McAllister et al., 1995, 1996; Horch et al., 1999); TTX did not prevent enhancement by BDNF of nonpyramidal cell dendritic growth, but does prevent some of the effects of BDNF on pyramidal cells (McAllister et al., 1996); and BDNF-induced dendritic branches were stable for up to several weeks in nonpyramidal cells, but are highly unstable in pyramidal neurons (Horch et al., 1999). Together, these comparisons suggest that the effects of BDNF on pyramidal and nonpyramidal neurons are qualitatively different, possibly because these two classes of cells express different complements of TrkB receptors or different downstream effectors of TrkB activation.

Depolarization promotes dendritic growth in nonpyramidal neocortical interneurons

We found that depolarizing neurons by adding 10 mM KCl to our organotypic cultures enhanced dendritic growth and branching of nonpyramidal interneurons to a level similar to that induced by 200 ng/ml BDNF, whereas blocking action potentials with TTX prevented dendritic growth. Therefore, these data suggest that dendritic growth of cortical interneurons in organotypic cultures requires action potentials and can be enhanced by depolarization. KCl-induced enhancement and/or TTX-induced reduction of dendritic length and complexity have been demonstrated previously in some systems (Reitstetter and Yool, 1998; Mertz et al., 2000; Vaillant et al., 2002), but not in others (Riccio and Matthews, 1987; Dalva et al., 1994; Rajan and Cline, 1998). Notably, TTX- or KCl-induced changes in dendritic length were not observed in dissociated cortical neurons *in vitro* (Kossel et al., 1997; Ramakers et al., 1998). If, as we propose below, the effect of depolarization was mediated by extracellular BDNF, then the failure to observe this effect in dissociated cultures could be attributable to the lower density of neurons in these cultures compared with organotypic slices and to dilution of the released BDNF to ineffective levels.

Depolarization effects are mediated by BDNF released by pyramidal neurons

Depolarization can promote dendritic growth in a cell-autonomous manner, e.g., by activating voltage-gated calcium channels, followed by calcium-dependent phosphorylation of the dendritic protein MAP2 (microtubule-associated protein 2) (Quinlan and Halpain, 1996; Sanchez et al., 2000; Wu et al., 2001; Vaillant et al., 2002). Alternatively, depolarization can enhance dendritic growth through cell–cell signaling (Matsutani and Yamamoto, 1998; Nedivi et al., 1998). The most parsimonious explanation of our results is that activity-dependent dendritic growth in our experiments was mediated by cell–cell signaling via BDNF, because the KCl-induced growth was prevented by antibodies to BDNF; however, it is difficult to rule out the possibility that KCl acted via a cell-autonomous pathway, and that BDNF provided only permissive conditions for the activation of this pathway.

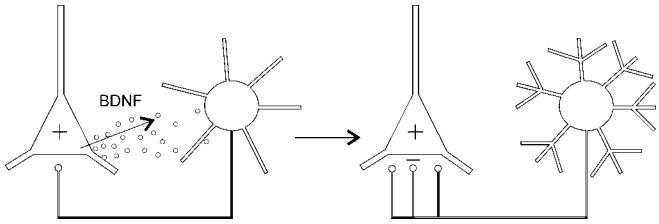

Figure 8. BDNF mediates a feedback loop that maintains a balance between excitation and inhibition. Left, A pyramidal neuron is assumed to be depolarized (+), causing it to increase its firing rate and thereby release more BDNF into the extracellular space, acting on TrkB receptors in a nearby inhibitory neuron. (Although this figure implies that BDNF is released from dendrites, BDNF may also be released from axon terminals.) Right, The released BDNF caused the inhibitory interneuron to ramify its dendritic and axonal arbors and thereby increase its inhibitory effect on the pyramidal cell (−) counteracting the initial depolarization.

In the cerebral cortex, BDNF is expressed only by pyramidal neurons, but TrkB, the preferred receptor for BDNF, is found in both pyramidal cells and GABAergic interneurons (Cellerino and Maffei, 1996; Rocamora et al., 1996; Gorba and Wahle, 1999). BDNF expression and release is induced or enhanced by excitatory synaptic and electrical activity, as has been consistently demonstrated *in vitro* (Zafra et al., 1990; Ghosh et al., 1994; Wetmore et al., 1994; Goodman et al., 1996; Gorba et al., 1999; Balkowiec and Katz, 2002) and *in vivo* (Isackson et al., 1991; Castren et al., 1992; Suzuki et al., 1995; Rocamora et al., 1996; Yan et al., 1997; Rossi et al., 1999). We therefore propose that depolarization-induced synthesis and release of BDNF from pyramidal neurons, either from dendrites (Davies, 1996; Haubensak et al., 1998; Hartmann et al., 2001; Kojima et al., 2001) or from axon terminals (Fawcett et al., 1998; Fawcett et al., 2000; Kohara et al., 2001), activated TrkB receptors on nearby GABAergic neurons and promoted their dendritic growth. In addition, it is possible that depolarization also enhanced TrkB expression on the recipient interneurons themselves (Tongiorgi et al., 1997; Meyer-Franke et al., 1998), causing them to be more responsive to ambient levels of BDNF.

A larger dendritic tree is likely to receive more axodendritic (mostly excitatory) synapses and thus increase electrical activity in the neuron. Moreover, the larger dendritic trees were most likely accompanied by larger (or denser) axonal arbors, as shown previously in dissociated cultures (Vicario-Abejon et al., 1998), although, in our slice cultures, it was difficult to image the axonal arbor in its entirety; a larger axonal arbor is likely to make more inhibitory synapses on postsynaptic targets. Thus, the depolarization-induced increase in network activity would be followed by an increase in synaptic inhibition, bringing activity levels back down. This negative feedback loop is illustrated schematically in Figure 8.

BDNF and activity-dependent maturation of GABAergic function

Electrical and synaptic activity *in vitro* has been shown previously to promote, and blockade of this activity, to reverse, an increase in the level of expression of GABA and GABAergic markers (Marty et al., 1996b; Rutherford et al., 1997) and an increase in the density of GABAergic synapses (Marty et al., 2000; Seil and Drake-Baumann, 2000). Similar to the effects demonstrated here, in the same studies these effects of activity were reproduced by exogenous BDNF and prevented by BDNF blockers, and/or, conversely, the effects of activity blockade were reproduced by BDNF blockers and prevented by exogenous BDNF, indicating that activity exerts its effects via BDNF–TrkB signaling. Various other aspects of GABAergic maturation were shown to be dependent on or enhanced by BDNF *in vitro* (Seil et al., 1994; Widmer and Hefti, 1994; Murphy et al., 1998; Yamada et al., 2002) and *in vivo* (Nawa et al., 1994; Huang et al., 1999; Aguado et al., 2003). Thus, BDNF may play a major role in regulating the maturation of the GABAergic inhibitory system and may act during development to keep GABAergic inhibition in step with the level of excitatory activity, thereby maintaining activity homeostasis in the network (Marty et al., 1997; Bolton et al., 2000; Turrigiano and Nelson, 2000). In the adult, BDNF may also mediate the effects of other physiological parameters, such as hormonal state, on dendritic morphology (Barbany and Persson, 1992; Toran-Allerand, 1996; Murphy et al., 1998). Finally, in addition to its global effect on the balance between excitation and inhibition, BDNF could also act very locally, by virtue of its anterograde or retrograde release and uptake in synapses (Altar et al., 1997; Fawcett et al., 1998; Hartmann et al., 2001; Kohara et al., 2001) and the dendritic targeting of its receptor protein and mRNA (Fryer et al., 1996; Tongiorgi et al., 1997), to promote growth of dendritic branches receiving active synapses at the expense of branches opposite less active synapses. Thus, BDNF could mediate dynamic sculpting of dendritic fields, providing a morphological substrate for activity-dependent plasticity of neuronal circuits, both during development and in adult learning.

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