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TrkB Signaling Modulates Spine Density and Morphology Independent of Dendrite Structure in Cultured Neonatal Purkinje Cells

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Neurotrophins cooperate with neural activity to modulate CNS neuronal survival and dendritic differentiation. In a previous study, we demonstrated that a critical balance of neurotrophin and neural activity is required for Purkinje cell survival in cocultures of purified granule and Purkinje cells (Morrison and Mason, 1998). Here we investigate whether TrkB signaling regulates dendrite and spine development of Purkinje cells. BDNF treatment of purified Purkinje cells cultured alone did not elicit formation of mature dendrites or spines. In cocultures of granule and Purkinje cells, however, continuous treatment with BDNF over a 2 week postnatal culture period increased the density of Purkinje cell dendritic spines relative to controls without causing a shift in the proportions of headed and filopodia-like spines. The increase in spine number was blocked by adding TrkB-IgG to the medium together with BDNF. Although BDNF alone did not consistently modify the morphology of dendritic spines, treatment with TrkB-IgG alone yielded spines with longer necks than those in control cultures. None of these treatments altered Purkinje cell dendritic complexity. These analyses reveal a role for TrkB signaling in modulating spine development, consistent with recently reported effects of neurotrophins on synaptic function. Moreover, spine development can be uncoupled from dendrite outgrowth in this reductionist system of purified presynaptic and postsynaptic neurons.

Key words: Purkinje cell; granule cell; cerebellum; neurotrophins; BDNF; TrkB; spines; dendrites

Dendritic spines receive >90% of all CNS excitatory synapses (Gray, 1959; Harris and Kater, 1994), making them excellent models for synaptogenesis and for short- and long-term synaptic modifications. Recent imaging advances have contributed to a renaissance in the study of dendrite and spine structure, function, and plasticity, in both static (Fletcher et al., 1994; Harris and Kater, 1994; Papa et al., 1995; McAllister et al., 1995, 1996, 1997; Murphy and Segal, 1997) and living preparations (Yuste and Denk, 1995; Yuste and Tank, 1996; Dailey and Smith, 1996; Ziv and Smith, 1996; Potter et al., 1997; Wilson Horch and Katz, 1997).

The molecular mechanisms underlying afferent-induced dendrite development and plasticity are beginning to emerge. Afferent contacts *in vivo* can trigger target cell dendritic extension and spine formation (Berry and Bradley, 1976a; Hillman, 1988; Purves et al., 1988), predicting a role for synaptic activity in spine and synapse development. Neurotrophins regulate innervation density (Causing et al., 1997) and dendrite and axon structure (Cohen-Cory and Fraser, 1995; McAllister et al., 1995, 1997; Cabelli et al., 1997). Neurotrophins cooperate with neural activity in regulating dendrite and spine outgrowth (McAllister et al., 1996) and also modulate synaptic transmission (Patterson et al., 1996; Stoop and Poo, 1996; Kang et al., 1997; Schuman, 1997; Wang and Poo, 1997). Other modulators of spine formation and dendritogenesis include estradiol (Woolley et al., 1997; Murphy et al., 1998) and signaling pathways involving CamKII (Wu and Cline, 1998) and Rac and Rho (Luo et al., 1996; Threadgill et al., 1997). Although these experiments provide clues to signals that drive synapse modification, the complete pathways from extrinsic cues to spine emergence and assembly of synaptic components are still unknown.

The cerebellar Purkinje cell is a good model for CNS synapse formation. Its development, connectivity, and synaptic plasticity are well characterized (for review, see Larramendi, 1969; Palay and Chan-Palay, 1974; Mason et al., 1990; Chedotal and Sotelo, 1992; Morrison and Mason, 1998). Experimental perturbations and mutant animal models implicate granule neuron afferents as a potent influence on Purkinje cell dendrite and spine development (for review, see Baptista et al., 1994). Our laboratory has developed methods to purify and coculture Purkinje cells with granule neurons, allowing interactions between these two cell types to be studied *in vitro* (Hatten, 1985; Baptista et al., 1994). Purkinje cells cultured alone extend axons but do not develop mature dendrites. Addition of purified granule neuron afferents is sufficient to drive dendrite and spine development of purified Purkinje cells (Baptista et al., 1994), raising the question of what signals granule cells provide for Purkinje cell differentiation.

Neurotrophins are attractive candidate regulators of Purkinje cell dendrite and spine development. Purkinje and granule cells both express TrkB, and BDNF promotes survival of purified

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Purkinje cells (for review, see Lindholm et al., 1997; Morrison and Mason, 1998). In a previous study documenting a critical balance of neurotrophin and neurotransmitter signaling required for Purkinje cell survival, we noted that although purified Purkinje cells treated with BDNF do not extend mature dendrites or spines, Purkinje cell spine density in cocultures with granule cells seems to increase with BDNF treatment (Morrison and Mason, 1998).

Here, we further analyze the effects of TrkB signaling perturbations on Purkinje cell dendrite and spine formation *in vitro*. Exogenous BDNF increases spine density without altering overall dendrite structure or spine morphology, whereas TrkB-IgG changes spine morphology. This culture system allows us to study dendrite and spine development separately, facilitating further analyses of neurotrophin modulation of neuronal morphology and synaptic function.

MATERIALS AND METHODS

Animals

Experiments were performed with C57BL/6J mice from a timed pregnancy breeding colony, with the plug date considered embryonic day 0 (E0). For any single Purkinje cell purification experiment, 30 postnatal day 0 (P0) pups were used, but occasionally it was necessary to use some P1 pups as well. Granule neurons were purified from pups on postnatal day 4.

Cultures and substrates

The cultures used for morphometric analysis were the same as those in a study on the effects of neurotrophins on cell survival (Morrison and Mason, 1998). Methods for preparation of cultures are summarized here briefly. Serum-free medium was composed of Eagle's basal medium with Earle's salts (Life Technologies, Gaithersburg, MD) supplemented with bovine serum albumin (10 mg/ml; A-9418, Sigma, St. Louis, MO), glutamine (2 mM, Life Technologies), glucose (32 mM), penicillin–streptomycin (29 U/ml each, Life Technologies), and Sigma I-1884 supplement (1:100 dilution, giving final concentrations of 5 ug/ml insulin, 5 ug/ml transferrin, and 5 ng/ml sodium selenite). Serum-containing medium was composed of Eagle's basal medium with Earle's salts, glutamine, glucose, penicillin–streptomycin, and 10% horse serum (Life Technologies).

Culture surfaces were pretreated overnight at 4°C with high molecular weight (>300,000 kDa) poly-D-lysine (500 ug/ml; Sigma or Specialty Media), and washed three times with distilled water before use.

Granule–Purkinje cocultures. Cerebellar granule neurons were purified as previously described (Hatten, 1985; Baird et al., 1992; Hatten et al., 1997; Morrison and Mason, 1998). Briefly, cerebella were collected and dissociated with trypsin, then spun through a two-step Percoll gradient. The dense cell fraction at the interface between the 35 and 60% Percoll phases was collected, and non-neuronal cells were removed by two sequential platings on Petri dishes precoated overnight with poly-D-lysine (100 ug/ml; Sigma). Nonadherent, neuronal cells were collected, centrifuged at 1100 rpm for 5 min, counted, and plated into poly-D-lysine coated Lab-Tek wells at 300,000 cells per well (this corresponds to 11×10^5 cells/cm^2). Cultures purified in this way consisted of ~95% granule cells and typically contained <5% of GFAP-positive cells. Granule cells were plated first in serum-free medium, incubated overnight, and Purkinje cells were added on the following day.

Purkinje cells were purified as previously described (Baptista et al., 1994; Hatten et al., 1997; Morrison and Mason, 1998). Briefly, a fraction enriched for Purkinje cells was obtained by passing papain-dissociated cerebellar cells over a 35% Percoll cushion. Astroglia were removed from this fraction by negative immunopanning using anti-GD3, and Purkinje cells were selected by positive immunopanning using anti-Thy-1.2, then removed from the surface with trypsin. The trypsin was inactivated by adding horse serum-containing medium or ovomucoid trypsin inhibitor, and the cells were centrifuged and resuspended in serum-free medium, counted, and plated at a density of 30,000 cells per Lab-Tek well (Nunc, Roskilde, Denmark; this corresponds to 1×10^5 cells/cm^2). Cultures prepared in this way consisted of 85–95% calbindin-D_{28k}-positive Purkinje cells.

Culture medium was changed every 3–4 d over a 14 d period.

Neurotrophins and TrkB-IgG. BDNF, NT-3, and TrkB-IgG were generously provided by Dr. G. Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, NY). NGF was a gift from Dr. Lloyd Greene (Columbia University Department of Pathology). Dose–response curves were generated for BDNF, NT-3, and NGF as described (Morrison and Mason, 1998). Experiments were designed to include concentrations of growth factor that gave maximal Purkinje cell survival at 14 d *in vitro* (DIV). Final concentrations of each factor were: BDNF, 10 ng/ml; NT-3, 50 ng/ml; NGF, 10 ng/ml; and TrkB-IgG, 25 ug/ml. Factors were added 1.5 hr after Purkinje cell plating on granule cell monolayers, and replaced when culture medium was changed.

Table 1. BDNF does not alter gross Purkinje cell differentiation in granule–Purkinje cell cocultures at 14 d *in vitro*

Treatment	Category 1[a]	Category 2[b]	Category 3[c]
Control	96.6% (904)[d]	1.1% (10)	2.3% (22)
BDNF	94.2% (228)	4.6% (11)	1.2% (3)

[a]Purkinje cells with normally developing dendritic processes and spines.

[b]Purkinje cells with small somata and long, thin neurites without spines.

[c]Purkinje cells with large somata and broad cytoplasmic lamellae, covered with spine-like structures (aberrant dendritic forms; see Baptista et al., 1994).

[d]Numbers in parentheses are number of cells with each general morphology.

Immunocytochemistry. Purkinje cells were visualized as described (Baptista et al., 1994; Morrison and Mason, 1998), by fixation with 4% paraformaldehyde and immunostaining with a rabbit polyclonal antibody against calbindin-D_{28k} (SWant, Bellinzona, Switzerland; 1:2000 final dilution). This marker specifically labels Purkinje cells within the cerebellum (Wassef et al., 1985; Christakos et al., 1987). Immunoperoxidase methods were used for ease of visualization of dendrites and spines.

Analysis

Experimental groups. Granule–Purkinje cocultures were cultured either untreated or treated with BDNF, TrkB-IgG, or both. Dendritic complexity, spine density, and spine morphology were quantitated for each experiment.

Cell sampling. Purkinje cells were observed using a Zeiss Axiophot or Leitz Dialux microscope with 20× or 100× objectives. For each experimental condition, nine Purkinje cells per well were selected for morphometric analysis by an unbiased, systematically randomized method based on their position within the well. In each well, nine visual fields were selected according to a plan that samples the entire well, with one field at the center and the remaining eight fields distributed evenly around the well between the center and the periphery. These fields were precisely determined for each well by setting standard coordinate axes. Each chosen visual field was 670 μm in diameter when viewed with a 20× objective on a Leitz Dialux microscope. Each of the nine visual fields was subdivided into thirty-two squares (100 μm on each side) which were numbered from 1 to 32, starting from the center and moving clockwise. Within each visual field, a well isolated Purkinje cell was selected in the square with the lowest number. If the square contained more than one Purkinje cell, then the square was subdivided into sixteen 25 μm squares, and Purkinje cells were chosen for analysis by a similar selection process.

Overall dendritic differentiation. To assess the effect of BDNF on overall dendritic differentiation, calbindin-immunopositive cells were classified into three categories (Table 1): (1) Normally developing Purkinje cells characterized by a large round soma and multiple dendritic processes covered with spines; (2) cells with a relatively small, irregular soma with long, thin bipolar neurites (these cells were thought to represent either immature Purkinje cells or cells with aberrant development); and (3) cells without dendritic processes, with a relatively large round soma and broad cytoplasmic skirt. Such cells were covered in spine-like structures and resembled forms seen in our previous study that developed on sparse beds of granule neurites (Baptista et al., 1994).

Analysis of spine density. Camera lucida reconstructions were made as stick figures ("skeletonized" drawings), representing the exact length and complexity of the dendritic arbor (see Fig. 2); these reconstructions were converted to digital files using a scanner. Spine density was determined on proximal and distal dendritic segments. Dendritic spines of Purkinje cells were defined as unbranched appendages protruding approximately at right angles from the dendritic shaft for a relatively short distance (<6 μm) (Papa et al., 1995). Proximal dendritic segments were considered as the dendritic segments between the first and second branch points. Distal

Table 2. Dendrite parameters of Purkinje cells in culture[a]

Treatment	n[b]	Combined dendritic length (μM)	Number of dendritic segments	Number of branch termination tips	Number of stem dendrites	Maximal branch order
Control	27	287.7 ± 175.9[c]	34.2 ± 17.8	20.7 ± 9.4	5.2 ± 1.9	5.7 ± 2.4
BDNF	27	252.8 ± 108.6	29.7 ± 12.2	18.2 ± 6.3	4.8 ± 1.6	5.3 ± 1.7
BDNF + TrkB-IgG	27	248.9 ± 117.4	29.6 ± 13.4	18.6 ± 7.1	5.0 ± 1.3	5.5 ± 2.1
TrkB-IgG	27	276.1 ± 139.5	30.2 ± 14.9	18.4 ± 8.2	4.3 ± 1.6	6.0 ± 2.8

[a]None of the experimental treatments had a statistically significant effect on any dendrite parameter.

[b]n = number of cells.

[c]Mean ± SD.

dendritic segments included the dendritic segments distal to the final branch point as well as the length between the penultimate and last branch points.

Spines were divided into two categories: (1) spines with heads and (2) filopodia-like, headless spines (see Fig. 6 for schematic). The occurrence of these two distinct populations of spine types has been described in developing dissociated hippocampal neurons in culture (Papa et al., 1995). Spines with heads included stubby spines with no obvious neck, spines with a thin neck and round to flattened heads, and mushroom-shaped spines with a relatively thick neck (Peters and Kaiserman-Abramhof, 1970) (see Fig. 6). Filopodia-like spines were thin, of uniform caliber, and their length exceeded their width. Spines were classified as indicated on the skeletonized drawings, with the aid of a camera lucida, as shown in Figure 2.

Spine density (number per 10 μm segment) was calculated by dividing the number of spines by the length of the segment in micrometers and multiplying by 10. In addition, the percentage of filopodia-like spines was calculated for each dendritic segment (see Fig. 3). Spine numbers were assessed on 1530 dendritic segments, on the same cells analyzed for dendritic complexity.

Analysis of spine morphology. Five Purkinje cells per well were further used for analyses of spine morphology. High-contrast, high-resolution images of individual dendritic spines were obtained using differential interference contrast (DIC) light microscopy and video contrast enhancement methods (Allen et al., 1981; Shotton, 1988; Salmon et al., 1989; Inoue and Spring, 1997) using a Zeiss Axiovert 35 inverted microscope with a halogen light source and a 100× plan neofluor oil objective (NA, 1.3), condenser (NA 1.4), and slider with a factor of 1.6×. A video zoom adapter (Zeiss) was inserted, and the system was calibrated using a stage micrometer. The images were captured by a high-resolution camera (Hamamatsu C2400). The optimized video images were then processed (images averaged and background subtracted) using MetaMorph software (Universal Imaging, West Chester, PA).

Features of spine morphology were determined for dendritic spines on the proximal and distal dendritic segments. Only those spines that were clearly visible, well separated from adjacent spines, and recognized by optical sectioning to emerge laterally from the dendritic shafts were measured (Papa et al., 1995; Inoue and Spring, 1997). For filopodia-like spines, the length from base to tip was measured on the monitor screen using the Image-1 program. For dendritic spines with heads, the neck length was measured, and the maximum and minimum diameter of the head were determined (see Fig. 6), the latter using the caliper function of the Image-1 system (Universal Imaging). The numbers of spines measured were 237 (control), 301 (BDNF-treated), and 225 (TrkB-IgG-treated).

Analysis of dendritic complexity. Dendritic stems were defined as segments of dendrite between the origin of the dendritic tree on the soma surface and the first branch point, intermediate dendritic segments fell between two successive branch points, and terminal dendritic segments were located between the last branch point and the branch termination tip. The length of individual dendritic segments on the scanned camera lucida drawings was measured using a computer-assisted image analysis system (Image-1; Universal Imaging). The following parameters of dendritic morphology were determined: (1) combined dendritic length, representing the sum of the length of all dendritic segments including stem, intermediate, and terminal dendritic segments; (2) the total number of dendritic segments; (3) the total number of branch termination tips; (4) the total number of dendritic stems; and (5) maximal branch order. Branch order was defined centrifugally from the soma; the first branch order represented the origin of the stem dendrite from the soma. The second branch order represented branch points arising from the stem dendrite, moving distally from the soma, and so on. One hundred and eight Purkinje cells selected from 12 wells, nine cells from one well of each experiment, were examined for analyses of dendritic complexity (Table 2).

Statistical design. Five parameters of dendritic complexity, two parameters of spine numbers, and four parameters of spine morphology were separately analyzed by two-way or three-way ANOVA using General Linear Models procedures of the SAS^R system (SAS Institute Inc.). Because the means and SDs for some of these parameters seemed to vary from experiment to experiment, we performed statistical tests to determine whether the data should be combined across experiments. In SAS parlance, we tested for the effects of experiments and for interactions between treatments and experiments. If the interaction between treatments and experiments was significant, data were not pooled across experiments, and each experiment was presented separately. If the effects of the treatments themselves were significant, then *post hoc* comparisons were made according to Dunnett's procedure, and treatment means were compared with the control mean in the same experiment.

For dendritic complexity, the statistical interaction between treatments and experiments was not significant for any dendritic parameter, so the data were collapsed across three experiments (Table 2).

For spine numbers, the interaction between treatments and proximal versus distal dendritic segments was not significant in either parameter, so the data were collapsed across these dendritic segments. However, because the interaction between treatments and experiments was significant, data from each experiment were analyzed and presented separately (see Fig. 3).

For spine morphology, in the majority of experiments, the main effect of treatments was significant for all four parameters ($F_{(2,745)} = 20.3, p < 0.001$ for maximum head diameter; $F_{(2,745)} = 5.3, p < 0.01$ for minimum head diameter; $F_{(2,745)} = 55.0, p < 0.001$ for neck length; $F_{(2,550)} = 31.2, p < 0.001$ for length of filopodia-like spines). Because the interaction between treatments and proximal versus distal dendritic segments was not significant in any of these parameters, data were collapsed across dendritic segments. However, because the interaction between treatments and experiments was significant in all four parameters, data from each experiment were analyzed separately (see Fig. 6).

RESULTS

In an *in vitro* model based on coculture of purified Purkinje and granule neurons, we demonstrated previously that although Purkinje cells cultured alone survive poorly and do not form mature dendrites, granule neurons are potent regulators of Purkinje cell survival and differentiation (Baptista et al., 1994). In addressing which factors mediate granule neuron-induced Purkinje cell development, we observed that BDNF and non-NMDA glutamate receptors modulate Purkinje cell survival (Morrison and Mason, 1998). BDNF treatment improves survival of purified Purkinje cells but is not sufficient to drive their dendritic development. In the present study, we investigate whether TrkB receptor signaling is involved in the differentiation of dendrites and/or formation of dendritic spines when Purkinje cells are cocultured with granule cells.

Exogenous BDNF, TrkB-IgG, or both were added to granule and Purkinje cell cocultures in serum-free medium 1.5 hr after

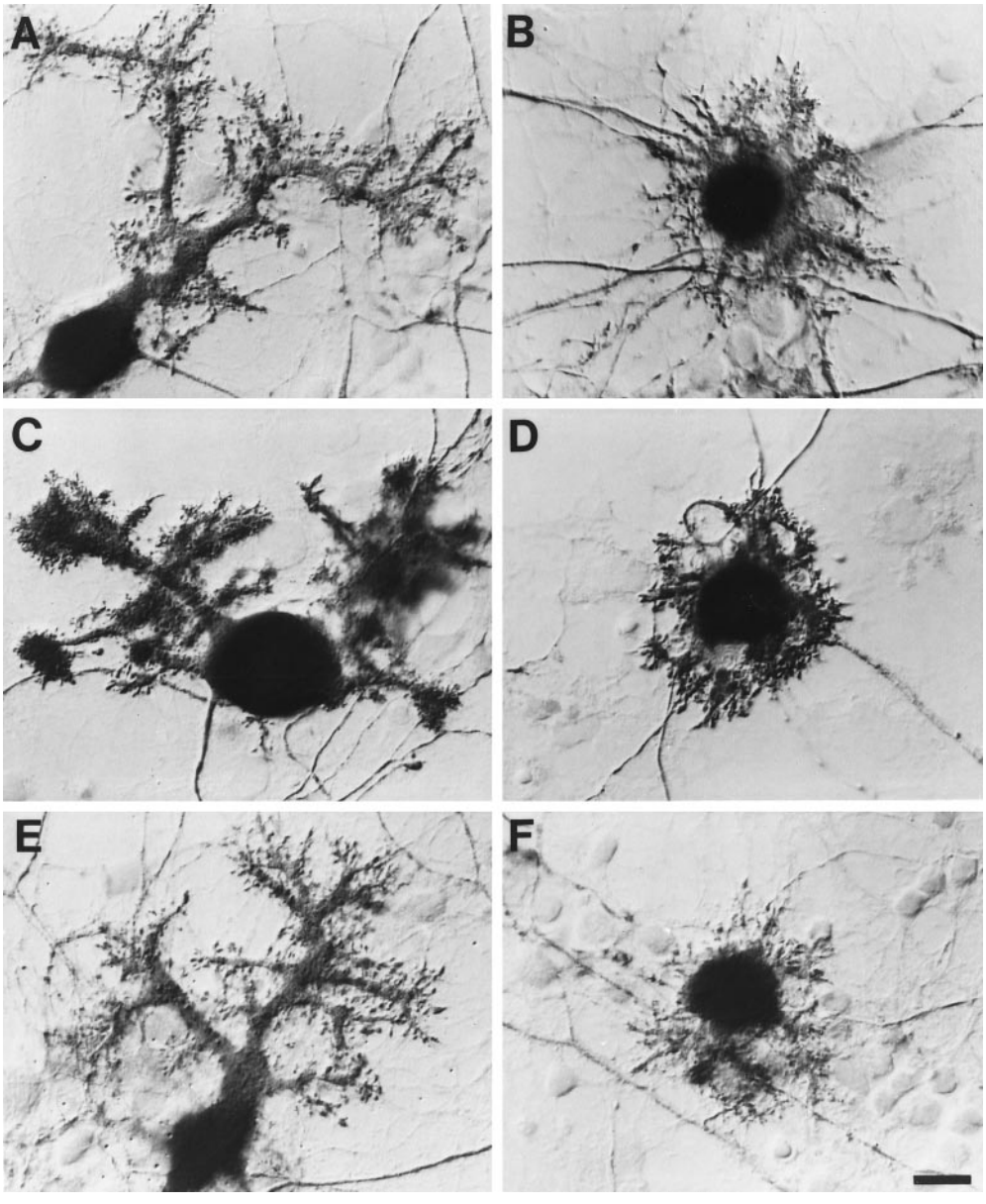

Figure 1. Purkinje cell dendrite and spine morphology *in vitro*. Purified Purkinje cells were cocultured with purified granule cells for 14 DIV in serum-free medium and immunostained with antibodies to calbindin-D_{28k}. *A*, *B*, Control cells (untreated); *C*, *D*, treated with BDNF; *E*, *F*, treated with TrkB-IgG. For each condition, examples of cells with well developed (*A*, *C*, *E*) and less well developed (*B*, *D*, *F*) dendrites are shown. Even at this relatively low magnification, an increase in spine density is apparent after BDNF treatment (*C*, *D*). Scale bar: *F*, 10 μm.

plating and each time the medium was changed. After 14 d *in vitro*, spine numbers, spine morphology, and dendrite structure were assessed. This time point was chosen because dendrites and spines are both actively developing by the second week *in vitro*. Headed spines are not found on the Purkinje cells at earlier times (6–7 d *in vitro*) with or without neurotrophin treatment. The cultures analyzed by morphometry in the present paper were the same as those in a previous study focusing on Purkinje cell survival (Morrison and Mason, 1998).

BDNF does not affect gross dendrite structure

BDNF treatment decreases the survival of Purkinje cells cultured with other cerebellar cells (Morrison and Mason, 1998), making it necessary to rule out the possibility that BDNF disrupts normal Purkinje cell development. To determine whether BDNF affected overall dendrite differentiation, general dendrite differentiation was examined in cultures grown with or without BDNF (Table 1). Calbindin-D_{28k}-positive Purkinje cells were divided into three categories (see Materials and Methods), with category 1 representing normally developing cells (forms seen during *in vivo* development) and categories 2 and 3 representing aberrant forms. The vast majority of cells displayed stages of development appropriate for 14 DIV, as described previously (see Materials and Methods; Table 1; Baptista et al., 1994). These cells had dendritic processes arranged in a multipolar manner or extended a single or double stem trunk emerging from one pole of the soma. The processes were covered with spines as *in vivo* (Fig. 1*A*,*B*). Only the normally developing cells in category 1 were analyzed further. In both the control cultures and those treated with BDNF, there was little difference in overall dendritic differentiation. Both groups, however, displayed a considerable range in the extent of dendritic development, from short processes with one or two branch points (Fig. 1*B*,*D*,*F*), to dendritic arbors with up to 14 branch points (Fig. 1*A*,*C*,*E*). Therefore, despite the deleterious effect of exogenous BDNF on Purkinje cell survival in granule–Purkinje cocultures, general Purkinje dendritic development appeared normal.

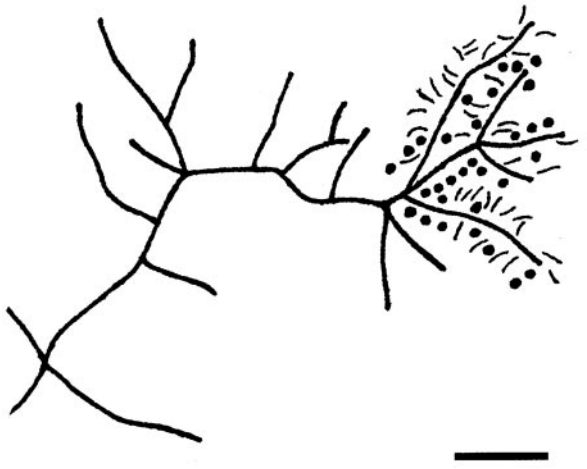

Figure 2. A stick figure camera lucida reconstruction of a part of the Purkinje cell dendritic arbor in Figure 1*A*. This skeletonized drawing represents the exact length and complexity of the dendritic arbor. All visible dendritic spines on the distal dendritic segments are schematically illustrated (*dots*, headed spines; *dashes*, filopodia-like spines). Scale bar, 10 μm.

BDNF modulates spine density

Qualitative observations suggested that Purkinje spine density and morphology might be affected by BDNF (Fig. 1; Morrison and Mason, 1998). To test this hypothesis, we determined the density of total spines and the percentage of filopodia-like spines for each experimental group. Data were collected separately for spines on proximal and distal dendrite segments (see Materials and Methods). In cultures treated with BDNF, the density of total dendritic spines was significantly increased in all three experiments ($F_{(3,1506)} = 49.8$, $p < 0.001$) (Figs. 1*A–D*, 2, 3). The increase in spine density compared with the control group ranged from 24 to 55% and was 42% on average. The increase in spine density caused by BDNF was blocked with the addition of TrkB-IgG (Fig. 3). TrkB-IgG alone, however, had no significant effect on spine density (Fig. 3). No difference was detected between spine densities on proximal and distal dendrites, so the proximal and distal data were combined (Fig. 3). Thus, exogenous BDNF increased the absolute number of total spines per Purkinje cell.

We next examined the proportion of filopodia-like and headed spines. The former are thought to represent immature spines, whereas the latter appear to be more mature, although it is not clear whether all spines on all neurons must progress from filopodial to headed forms (Papa et al., 1995; Dailey and Smith, 1996; Ziv and Smith, 1996). Filopodia-like spines comprised 43.9% of all dendritic protrusions in control cultures (Fig. 3). This percentage was not consistently changed by treatment with BDNF, TrkB-IgG, or both (Fig. 3). The percentage of filopodia-like spines was not significantly higher on the distal dendritic segments (44.7%) than on proximal segments (40.8%) (data not shown). Thus, although BDNF treatment increased spine density, it did not change the steady-state relative proportion of filopodia-like to headed spines, a possible index of spine maturity.

Because neurotrophins can signal through several receptors, we further explored the receptor specificity of spine density regulation in our system. Granule–Purkinje cocultures treated with NGF (a TrkA ligand) or with NT-3 (primarily a TrkC ligand) did not display Purkinje cell spine densities significantly different from those in untreated control cultures (Fig. 4). The BDNF-induced increase in spine density was therefore most likely mediated by signaling through the TrkB receptor.

TrkB perturbation alters spine morphology

Initial qualitative observations suggested that TrkB perturbations might alter spine morphology as well as spine number. To measure spine length, head diameters, and neck lengths, we adapted existing measurement methods to our culture setting. Of obvious concern was whether the spines were within the limits of resolution of our microscopic system. Since the 1980s, analog and digital video devices coupled to the light microscope have dramatically boosted image contrast, resulting in the ability to use objectives and condensers at much higher numerical aperture values than previously possible (Inoue, 1989). The limit of resolution for our DIC optical lenses with a high numerical aperture was 0.25 μm according to the Rayleigh criterion (Hecht, 1987). By enhancing the speed of image acquisition, subtracting unwanted optical noise, averaging out random electronic and photon noise, and decreasing the depth of field via video devices such as those used here, it was possible to observe and quantitate diffraction images of cell structures five to ten times smaller than the Rayleigh resolution limit (50–25 nm in our system). Together with the fact that the size of a pixel of our digitized video images corresponded to 59 nm when a 2× zoom attachment was used, our video devices rendered spines 0.5–3 μm in average size readily visible, such that the overall length and neck length were easily measurable.

Even with the above improvements in video-enhanced DIC image quality, measurement of the absolute distance between two edges was still difficult because of ambiguity in interpreting the position of edges of the diffraction images (Schnapp et al., 1988; Inoue, 1989), particularly during assessment of such parameters as the diameter of spine heads. To detect edges of spines more precisely on the monitor screen and to measure the diameter with the "caliper" function of the Image-1 system, a line intensity scan (Inoue, 1989) was performed across the images of the spine heads, and the edges were determined by locating the inflection points of the signal intensity curves (Fig. 5).

Using these methods, we measured the head diameters and neck length of headed spines and the total length of filopodial spines. When Purkinje cells were treated with exogenous BDNF, the morphology of individual dendritic spines showed no obvious or consistent changes compared with those of control cells for either class of spines (spines with heads vs filopodia-like spines) (Fig. 6). BDNF treatment therefore increased the density of dendritic spines without affecting the spine morphology apparent in these static preparations.

In contrast, in Purkinje cells grown with TrkB-IgG, the neck length of headed spines was significantly increased from 66 to 178% (average increase, 109%) relative to that in control cultures. This was consistent in all three experiments (Fig. 6). Changes in the diameter of spine heads after treatment with TrkB-IgG were not as consistent as the increases in neck length. The length of filopodia-like spines also did not change reproducibly with BDNF or TrkB-IgG treatment (Fig. 6). Thus, TrkB-IgG treatment results in a change in spine neck length, but no consistent changes in other parameters of spine morphology.

Neither BDNF nor TrkB-IgG affects dendritic complexity

Previous analyses using cortical slices indicate that neurotrophins modify both spine parameters and dendrite morphology simultaneously, raising the question of whether BDNF modulation of

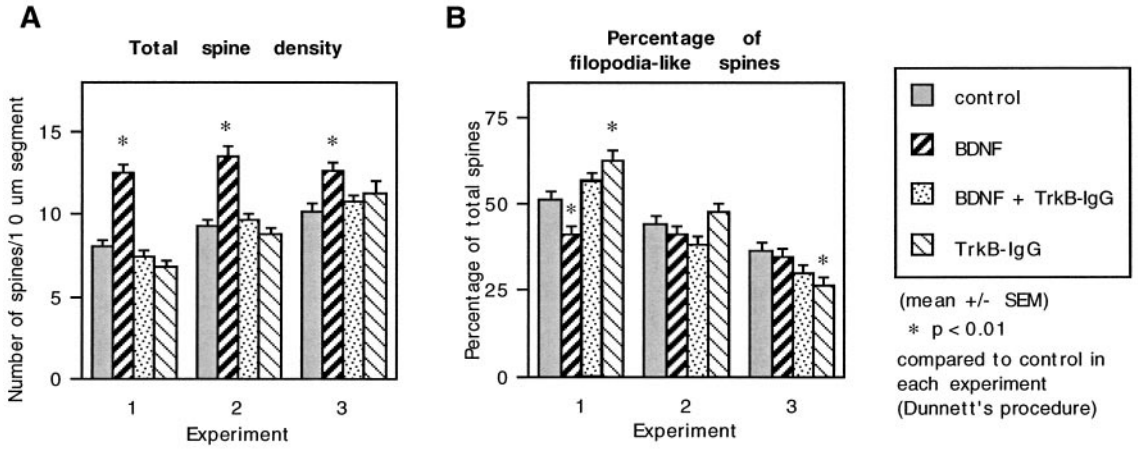

Figure 3. TrkB signaling and Purkinje cell spine density. *A*, Spine density. The BDNF-induced increase in spine density is highly significant ($p < 0.001$). *B*, Percentage of filopodia-like spines. Number of dendritic segments analyzed for each bar: *Experiment 1*: *control*, $n = 145$; *BDNF*, $n = 130$; *BDNF + TrkB-IgG*, $n = 142$; *TrkB-IgG*, $n = 114$; *Experiment 2*: *control*, $n = 122$; *BDNF*, $n = 129$; *BDNF + TrkB-IgG*, $n = 126$; *TrkB-IgG*, $n = 137$; *Experiment 3*: *control*, $n = 126$; *BDNF*, $n = 129$; *BDNF + TrkB-IgG*, $n = 132$; *TrkB-IgG*, $n = 98$.

spine number and morphology is coordinately regulated with changes in dendrite morphology (McAllister et al., 1995). Although our general survey by inspection showed no dramatic differences in Purkinje dendrite development (Table 1), we investigated whether there could be more subtle differences in dendrite structure under the influence of BDNF.

Quantitative analyses of dendrite parameters were designed with the aim of fairly sampling cultures that contain variations in cell geometry in a two-dimensional setting. Most quantitation of neuronal dendritic complexity *in vitro* has been performed on slices in which the circuitry is relatively intact (Studer et al., 1994; Bannister and Larkman, 1995; McAllister et al., 1995). In the few studies of dendritic complexity in dissociated neurons in culture, Sholl analysis or fractal analysis was used (Sholl, 1953; Neale et al., 1993; Le Roux and Reh, 1995; Nuijtinck et al., 1997). Although quantifying dendritic complexity by fractal dimensions can be useful in detecting the overall development of dendritic arbors during the first 4 d in culture, fractal dimensions failed to represent dendritic growth accurately after 4 DIV (Neale et al., 1993). Fractal analysis was therefore not used in the present study, in which subtle changes in dendritic complexity were investigated in relatively mature neurons (14 DIV). Moreover, although Sholl analysis is a well recognized method to quantitate overall extent of dendrite branching that provides information on branch number relative to distance from the soma, we chose to analyze five dendrite parameters separately. The geometric independence of these parameters facilitates identifying the components of dendritic complexity that might be affected by exogenous factors.

We measured combined dendritic length, total number of dendritic segments, number of branch termination tips, number of stem dendrites, and maximal branch order for Purkinje cells in coculture with granule neurons. There were no differences in any of these parameters between Purkinje cells grown in the presence of BDNF and those in control cultures (Table 2). Purkinje cells grown with TrkB-IgG alone or with BDNF and TrkB-IgG together also showed no significant difference in any of these dendritic parameters, compared with controls (Table 2). Thus, perturbation of TrkB signaling between granule and Purkinje cells by addition of BDNF or TrkB-IgG does not affect the overall extent of dendritic outgrowth or branching in this culture system.

DISCUSSION

The formation of mature Purkinje dendrites entails elaboration of a complex dendritic tree as well as regulation of spine maturation. The degree to which these two processes are interconnected is unclear. Previous experiments indicate that exogenous BDNF cannot elicit dendrite outgrowth from purified neonatal Purkinje cells cultured alone, whereas addition of purified granule neurons instigates Purkinje dendrite and spine development. The present results show that treatment with exogenous BDNF, TrkB-IgG, or both has no effect on dendritic complexity of purified Purkinje cells cocultured with their afferent granule cells. Despite the absence of effects on dendritic complexity in this culture setting, exogenous BDNF increases the density of dendritic spines without changing their shape or the proportion of spines with heads versus filopodia-like spines. In contrast, treatment with TrkB-IgG does not change spine density but produces spines with longer necks than those in control cultures. These results suggest that TrkB signaling can regulate spine development during granule–Purkinje cell interactions *in vitro* in a manner that is separable from effects on dendritic development.

Regulation of dendritic development

The sequence of dendritic and axonal process emergence and distribution of cytoskeletal elements during neuronal development has been characterized *in vitro* (Dotti et al., 1988; Baas et al., 1989; Le Roux and Reh, 1994). Extrinsic cues that influence dendritic development include extracellular matrix (Rousselet et al., 1990; Le Roux and Reh, 1994; Jeffery et al., 1996; but see Denis-Donini and Estenoz, 1988; Qian et al., 1992), hormones (Murphy et al., 1998; Stern and Armstrong, 1998), and growth factors (Lein et al., 1995; Prochiantz, 1995; Meyer-Franke et al., 1995; Withers et al., 1997). Altering environmental stimulation, and therefore neural activity, also changes dendritic development (Greenough and Volkmar, 1973; Greenough et al., 1973; Pysh and Weiss, 1979; Kleim et al., 1997), in agreement with newer data that direct modulation of glutamate receptors or ion channels affects dendritogenesis (Schilling et al., 1991; Bodnarenko et al., 1995; Metzger et al., 1998; Muller et al., 1998).

Our results did not implicate TrkB signaling in determining dendritic complexity of postnatal Purkinje cells cultured with their granule cell afferents, despite previous reports that neuro-

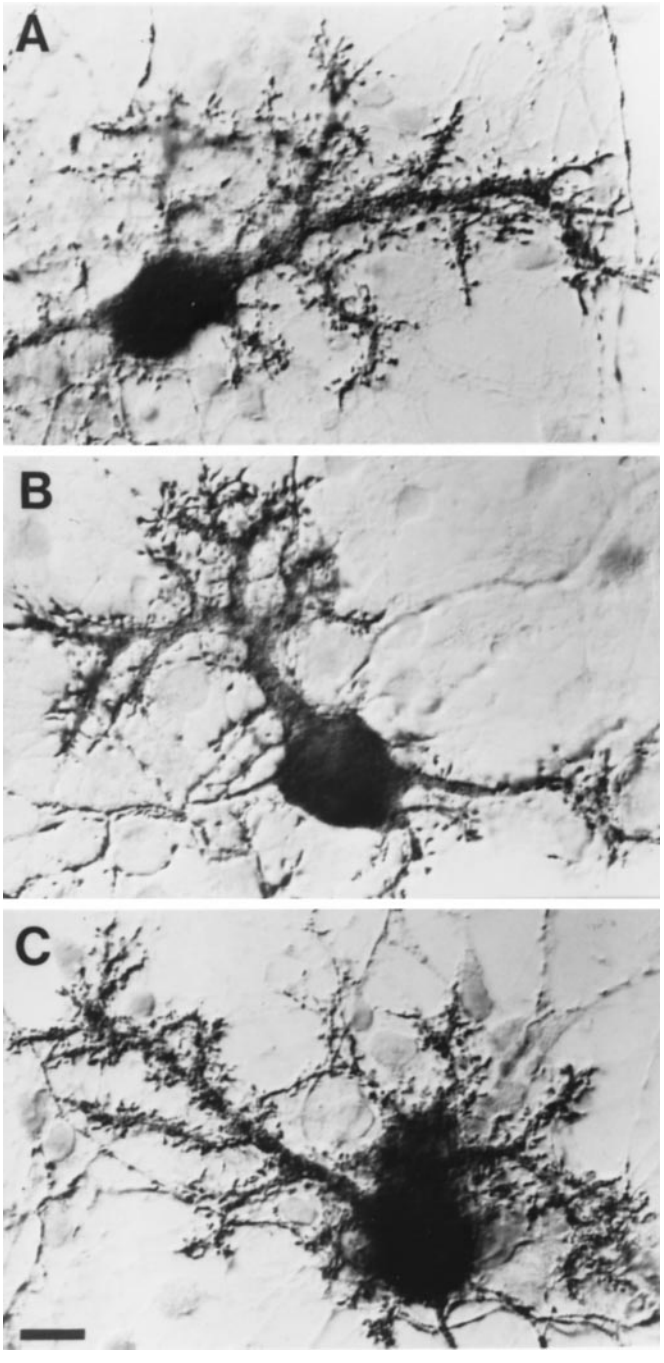

Figure 4. Other neurotrophins and Purkinje cell spine density. Purified Purkinje cells were cocultured with purified granule cells for 14 DIV in serum-free medium and immunostained with antibodies to calbindin-D_{28k}. *A*, Untreated control cell; *B*, treated with NGF; *C*, treated with NT-3. Neither of these neurotrophins increases spine density relative to controls. Scale bar: *C*, 10 μm.

trophins regulate Purkinje cell differentiation in other settings (Cohen-Cory et al., 1991; Lindholm et al., 1993; Mount et al., 1993, 1994; Schwartz et al., 1997). The present study differs from these in that the granule–Purkinje cocultures used here omit cellular components such as glia and other cells in the more intact intrinsic circuitry of brain slices (Banker, 1980; McAllister et al., 1995; Seil et al., 1995). In addition, previous studies used medium-containing serum, which alters the effects of neurotrophins (for review, see Morrison and Mason, 1998). Previous studies were also largely confined to earlier time points or to less mature stages in Purkinje cell development than those detailed here. Our approach has the advantage of pinpointing the action of reagents tested to a single presynaptic and postsynaptic cell pair. Coculture of purified granule and Purkinje cells allows analysis of dendritic development in a reductionist setting, such that neurotrophins or agents that perturb TrkB signaling act on these cells and not through other "bystander" cells. Modulators of granule neuron-induced Purkinje cell development identified using this system can be reexamined in more complex settings such as slices, in which perturbations of TrkB signaling may reveal additional effects on spines and/or dendrites.

Our experiments provide a counterpoint to two recent studies documenting BDNF regulation of dendritic development. First, neurotrophins added to postnatal cortical slices after the beginning of dendritogenesis produce extraordinary plasticity of dendrite and spine outgrowth (McAllister et al., 1996). In contrast, perturbing TrkB signaling (via BDNF or TrkB-IgG) in granule–Purkinje cocultures alters spine parameters without altering dendritic outgrowth, demonstrating that neurotrophins can regulate dendrite and spine development independently. Second, in BDNF knock-out mice, Purkinje cells display stunted dendrites (Schwartz et al., 1997), but in these mice, both granule and Purkinje cells develop in an environment lacking BDNF. In the present study, granule and Purkinje cells develop embryonically under the influence of BDNF, then at birth are cultured with exogenous BDNF or with TrkB-IgG and display dendritic outgrowth similar to controls. Together, these findings suggest that normal Purkinje cell dendritic development may require BDNF or TrkB prenatally, for effects on future dendritic structure (Schwartz et al., 1997), and postnatally, for effects on spine density (see below).

Regulation of spine development

In a minimalist setting comprised of presynaptic granule and postsynaptic Purkinje cells, TrkB signaling modulates Purkinje cell spine density and morphology independent of dendritic complexity. Known regulators of spine formation include neural activity (Pysh and Weiss, 1979; Chang and Greenough, 1984; Dalva et al., 1994; Hosokawa et al., 1995; Kossel et al., 1997; but see Harris and Kater, 1994; Sorra and Harris, 1998), neurotrophins (McAllister et al., 1996, 1997), hormones (Gould et al., 1990; Woolley et al., 1990; Danzer et al., 1998; Murphy et al., 1998), and combinations thereof (Cohen-Cory et al., 1991; Levine et al., 1995; McAllister et al., 1996). Dendritic growth and spine genesis may be either coregulated (McAllister et al., 1995, 1996, 1997) or separable (Dalva et al., 1994; Kossel et al., 1997). The present findings support the latter model; TrkB signaling can regulate Purkinje spine development and morphology without affecting overall dendrite morphology.

How does TrkB signaling affect Purkinje spine development? Ultrastructural analyses suggest that afferent contacts on dendritic shafts elicit spine eruption, but that spines can also form before afferent contact (Berry and Bradley, 1976b; Chang and Greenough, 1984; Landis, 1987; Harris and Stevens, 1989; Vaughn, 1989; Harris et al., 1992). Imaging studies have revealed dynamic interactions between afferents and dendritic filopodia, previously thought to be precursors of spines (Berry and Bradley, 1976b; Vaughn, 1989; Cooper and Smith, 1992; Papa et al., 1995), although filopodia can be present on subsequently spineless dendrites (Saito et al., 1992). Filopodial spines extend and retract,

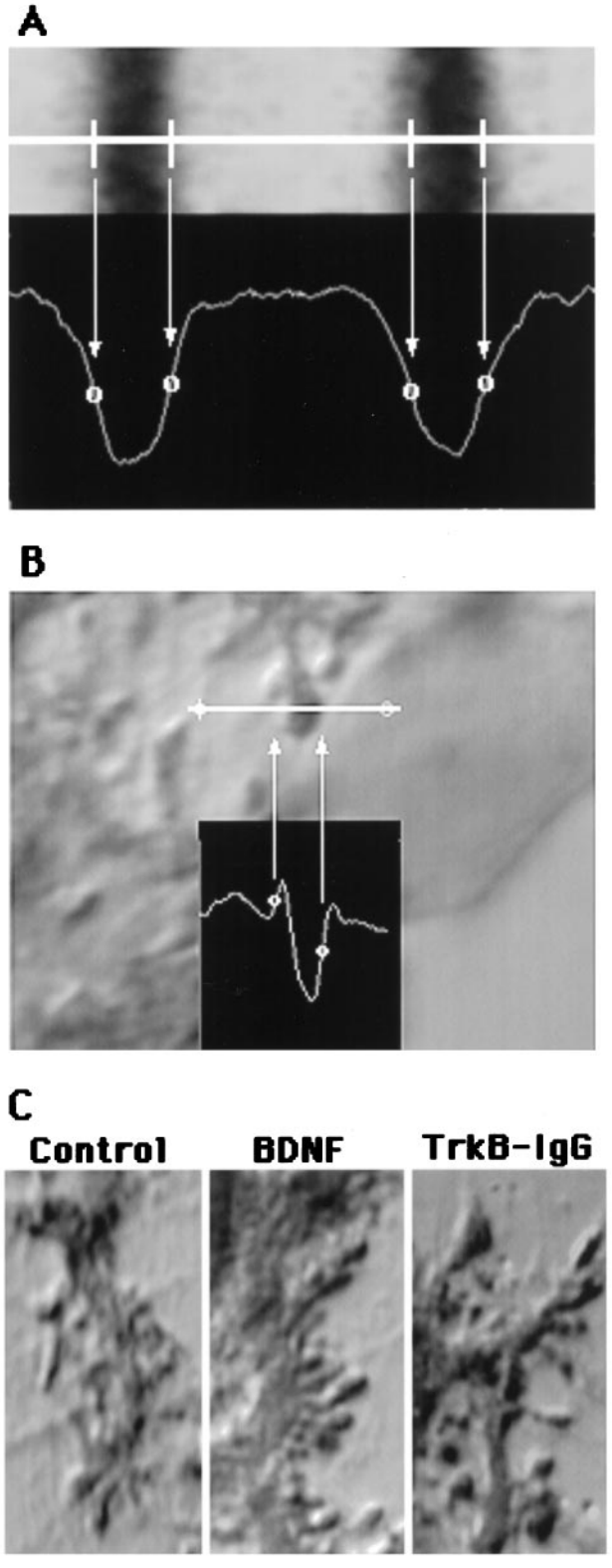

Figure 5. Edges of spines can be defined using a line intensity scan. A method for edge detection on video-enhanced DIC images. *A*, A stage micrometer is visualized on the monitor at high magnification using DIC optics and an image analyzer. Edges are readily determined by the naked eye as indicated by *vertical bars*. The *white horizontal line* indicates the line along which the signal intensity has been scanned, producing the intensity curve below. The edges of the stripes appear to correspond to the inflection points of the curves (*open circles*). *B*, In DIC images of dendritic spines at high magnification, it is frequently difficult to determine edges of individual spines. In these cases, edges were determined (*vertical arrows*) after a line intensity scan was performed and the inflection points (*open circles*) were determined on the signal intensity curve. *C*, Examples of video-enhanced DIC images used for morphometry of spines in *control*, *BDNF*-treated, and *TrkB-IgG*-treated cultures.

and the lifetime of individual spines increases with maturation (Dailey and Smith, 1996; Ziv and Smith, 1996). Dynamic imaging studies will be necessary to reveal how and when BDNF treatment increases spine density without changing the relative proportion of headed to filopodia-like spines (Fig. 3).

BDNF could act on the afferent/presynaptic granule neurite or directly on the postsynaptic Purkinje cell, because TrkB receptors are found on both cell types (Gao et al., 1995; Segal et al., 1995; Lindholm et al., 1997). BDNF could modulate granule neurite (parallel fiber) extension and contacts with Purkinje cells (for precedent, see Cohen-Cory and Fraser, 1995), which might in turn elicit spine emergence. Neurotrophins also regulate presynaptic transmitter release (Stoop and Poo, 1996; Wang and Poo, 1997), presynaptic configuration (Martinez et al., 1998), and synaptic function (Kang and Schuman, 1995; Figurov et al., 1996; Kang and Schuman, 1996; Patterson et al., 1996; Kang et al., 1997). If BDNF is released presynaptically (Altar et al., 1997), it could act on the postsynaptic cell to signal spine eruption. Consistent with this model, TrkB is localized in the postsynaptic density (C. P. Drake, E. R. Kandel, T. A. Milner, and S. L. Patterson, unpublished observations; Levine et al., 1995).

One apparent enigma is why BDNF increases spine number, whereas TrkB-IgG does not decrease spine number. Conversely, spine neck length is increased by TrkB-IgG, but unaffected by BDNF. The TrkB-IgG reagent is functional in binding exogenous BDNF, as demonstrated by the "neutralization" of BDNF effects when TrkB-IgG and BDNF are added together *in vitro* (Fig. 3) (Morrison and Mason, 1998). TrkB-IgG is also effective in altering endogenous signaling: it alters spine neck length (Fig. 6). TrkB-IgG may not fully block signaling by endogenous BDNF; BDNF may be released synaptically and therefore may be "protected" from TrkB-IgG access (Levine et al., 1995; Snider and Lichtman, 1996; Altar et al., 1997). Normal spine number may require a minimum threshold of BDNF signaling, and the TrkB-IgG reagent may not suppress endogenous BDNF signaling below that threshold. Finally, TrkB-IgG may also interfere with NT4 signaling as well as BDNF signaling, producing different effects than simple BDNF signaling perturbations.

What are the consequences of alterations in spine shape and number? The idea that changes in spine morphology underlie memory storage has a rich history (Ramon y Cajal, 1911; Hebb, 1949; Eccles, 1965). Previous models of spine conformation predicted that alterations of parameters such as neck diameter or length would alter conduction velocity and/or diffusion of metabolites for synaptic function (Rall and Rinzel, 1973; Harris and Stevens, 1988; Koch et al., 1992; Harris and Kater, 1994). Newer studies argue that although spine parameter changes probably have no effect on synaptic currents, they may affect the ability of the spine to act as a biochemical compartment (Yuste and Denk, 1995; Svoboda et al., 1996). Dendrite and spine abnormalities have been reported in many human pathologies, including retardation, epilepsy, and neurodegenerative disorders (Marin-Padilla, 1972; Purpura, 1974; Scheibel et al., 1974; Kreutzberg et al., 1997). These abnormalities include long spine necks and

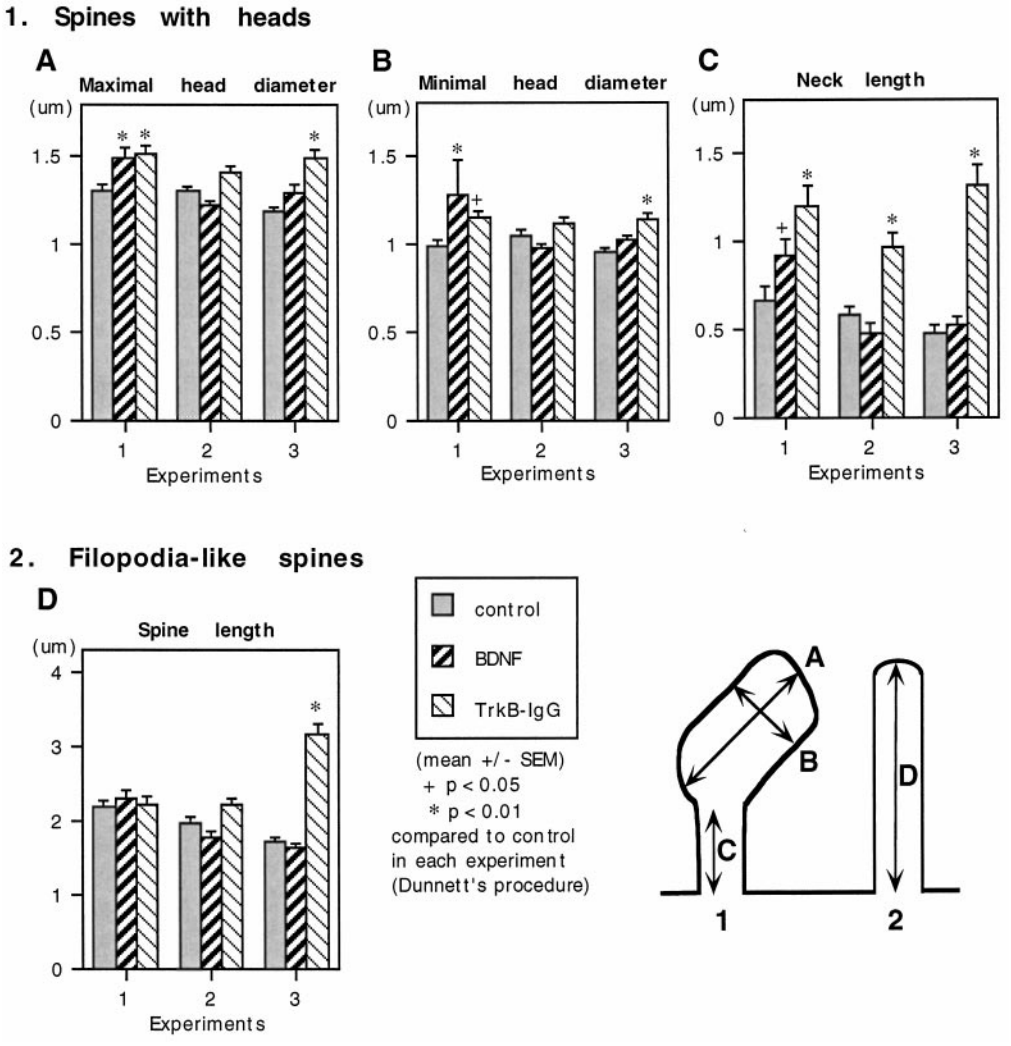

Figure 6. Morphological effects of TrkB signaling perturbations on Purkinje cell dendritic spines. *A–C*, Maximum and minimum head diameter and neck length are shown for spines with heads. Treatment with TrkB-IgG leads to a significant increase in neck length in all three experiments. *D*, Length of filopodia-like spines. Schematic indicates the parameters measured (*A–D*) for headed (1) and filopodial (2) spines. Number of dendritic spines measured for each bar: *A–C*, *Experiment 1*: *control*, $n = 77$; *BDNF*, $n = 56$; *TrkB-IgG*, $n = 70$; *Experiment 2*: *control*, $n = 86$; *BDNF*, $n = 129$; *TrkB-IgG*, $n = 78$; *Experiment 3*: *control*, $n = 74$; *BDNF*, $n = 116$; *TrkB-IgG*, $n = 77$. *D*, *Experiment 1*: *control*, $n = 69$; *BDNF*, $n = 63$; *TrkB-IgG*, $n = 67$; *Experiment 2*: *control*, $n = 54$; *BDNF*, $n = 92$; *TrkB-IgG*, $n = 70$; *Experiment 3*: *control*, $n = 56$; *BDNF*, $n = 53$; *TrkB-IgG*, $n = 44$.

prominent heads, similar to the features of Purkinje spines after perturbation of TrkB signaling with TrkB-IgG. An increase in spine numbers, even if they are the appropriate shape as in the BDNF-treated cells, could lead to overstimulation of the spine-laden neuron and may be related to the increased cell death when Purkinje cells in cocultures are continuously grown in the presence of BDNF (Morrison and Mason, 1998). Based on all of these observations, the changes in spine number and length caused by BDNF or TrkB-IgG treatment may reflect (or cause) altered synaptic function.

Conclusions

Purified granule–Purkinje cell cocultures permit uncoupling of spine development and dendrite outgrowth. In combination with ongoing studies of hippocampal and cortical neurons, this approach should reveal additional regulators of Purkinje cell differentiation, illuminating the full repertoire of signaling pathways during dendrite and spine development.

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