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The Actions of BDNF on Dendritic Spine Density and Morphology in Organotypic Slice Cultures Depend on the Presence of Serum in Culture Media

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Abstract

We have previously shown that brain-derived neurotrophin factor (BDNF) increases dendritic spine density and the proportion of stubby spines in apical dendrites of CA1 pyramidal neurons of hippocampal slice cultures maintained in serum-free media. We show here that serum withdrawal causes an increase in the proportion of thin spines and a decrease in the fraction of stubby spines, without changing the overall density of dendritic spines. When slices are maintained in serum-containing media, BDNF also increased spine density but had the opposite effect on spine morphology: it increased the proportion of mushroom and thin spines and decreased the proportion of stubby spines. Intriguingly, slices maintained in serum media showed a lower p75NTR-to-TrkB expression level than serum-free slices, even after BDNF exposure. The differential actions of BDNF on spine morphology depending on the presence of serum in culture media, together with the difference in neurotrophin receptor expression are reminiscent of opposing functional signaling by p75NTR and Trk receptors, and reveal a complex modulation of dendritic morphology by BDNF signaling.

Keywords

CA1 pyramidal neuron; biolistic transfection; hippocampus; confocal microscopy; enhanced yellow fluorescent protein; organotypic slice culture

The establishment of proper dendrite architecture is a fundamental process in nervous system development, where the formation of dendritic spines is critical for the formation of excitatory synaptic networks. The characteristic morphology of dendritic spines has been long postulated to correlate with their postulated function as biochemical or electrical compartments (Kasai et al., 2003; Shepherd, 1996). Based on their morphology, dendritic spines have been classically categorized into three major types: stubby (type-I), mushroom (type-II), and thin (type-III) spines (Peters and Kaiserman-Abramof, 1970; Peters and Kaiserman-Abramof, 1969). Differences in spine morphology have been postulated to play a fundamental role in synaptic plasticity models of memory formation and storage (Chapleau and Pozzo-Miller, 2007; Ethell and Pasquale, 2005; Nimchinsky et al., 2002; Yuste and Bonhoeffer, 2001). Several lines of

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evidence implicate neurotrophic factors in the formation and structural plasticity of existing dendritic spines in CNS pyramidal neurons (McAllister et al., 1999; Tyler et al., 2002a).

The mammalian neurotrophic factors, a family of growth factors that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5), have essential roles in neuronal survival and differentiation (Huang and Reichardt, 2001; Lewin and Barde, 1996). In addition to these classical functions, BDNF in particular has been shown to be one of the most potent modulators of synaptic transmission and plasticity, as well as neuronal and synaptic morphology (Amaral et al., 2007; Black, 1999; Poo, 2001; Tyler et al., 2002a; Vicario-Abejon et al., 2002). Each neurotrophin exerts its actions through binding and activation of specific, membrane-bound tropomyosin-related kinase (Trk) receptors, or a single pan-neurotrophin receptor, the so-called p75NTR (Barbacid, 1993). Individual Trk receptors have high affinity for specific neurotrophins: TrkA for NGF, TrkB for BDNF and NT-4, and TrkC for NT-3; on the other hand, all neurotrophins bind to p75NTR with equal affinity and no apparent selectivity (Chao and Hempstead, 1995). BDNF has been shown to modulate dendritic spine density and morphology in cortical pyramidal neurons (Horch and Katz, 2002; Horch et al., 1999; McAllister et al., 1996, 1997; McAllister et al., 1995), cerebellar Purkinje, basket and stellate neurons (Mertz et al., 2000; Shimada et al., 1998), dentate granule neurons (Danzer et al., 2002; Tolwani et al., 2002), olfactory bulb granule cells (Matsutani and Yamamoto, 2004), and hippocampal pyramidal neurons (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; Ji et al., 2005; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). These observations clearly demonstrate the role of BDNF on dendritic spine formation, but unfortunately they were obtained from different types of culture systems without considering the potential confounding actions of serum in culture media. Since the presence of serum in culture preparations has been shown to affect neurotrophin actions (Morrison and Mason, 1998; Tyler and Pozzo-Miller, 2003), we compared the effects of BDNF on spine density and morphology in apical dendrites of CA1 pyramidal neurons of hippocampal slice cultures maintained in serum-containing media *vs.* its actions in serum-free media. As we shown before, BDNF increased spine density and the proportion of stubby spines in slices kept in serum-free media (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). Even though it also increased spine density in slices maintained in serum-containing media, BDNF had the opposite effect on spine morphology, i.e. it increased the proportion of thin (type-III) spines while reducing the fraction of stubby (type-I) spines.

MATERIAL AND METHODS

ORGANOTYPIC SLICE CULTURES

All animal procedures followed national and international ethics guidelines and were reviewed and approved by the IACUC at UAB on an annual basis. Hippocampal slice cultures were prepared from postnatal-day 7 to 10 (P7-P10) Sprague-Dawley rats and maintained *in vitro* as previously described (Pozzo Miller et al., 1993; Stoppini et al., 1991; Yamamoto et al., 1989). Briefly, rats were quickly decapitated and their brains aseptically dissected and immersed in ice-cold dissecting solution, consisting of Hanks' Balanced Salt Solution (HBSS), supplemented with glucose (36mM) and antibiotics/antimycotics (1:100; penicillin/streptomycin/amphotericin B). Hippocampi were then dissected and transversely sectioned into ~500µm slices using a custom-made tissue slicer (Katz, 1987) strung with 20µm-thick tungsten wire (California Fine Wire Company; Groover Beach, CA). Slices were incubated at 4°C for ~30min, and then plated on tissue culture inserts (0.4µm pore size, Millicell-CM, Millipore Corporation; Billerica, MA). Culture media contained minimum essential media (MEM; 50%), HBSS (25%), heat-inactivated equine serum (20%), L-glutamine (1mM), and D-glucose (36mM). Slices were maintained in incubators set at 36°C, 5% CO₂, 98% relative

humidity. Culture medium was first changed at 4 days *in vitro* (div) and every 2 days afterwards. It should be noted that, even though organotypic slice cultures are isolated *in vitro* for extended times, CA1 pyramidal neurons continue to develop acquiring dendritic and spine morphologies that are not significantly different than those of CA1 neurons from age-matched acutely prepared hippocampal slices (De Simoni et al., 2003).

To avoid potential confounding effects due to unknown factors in the equine serum used in standard organotypic slice culture protocols, our prior studies used slices maintained in defined serum-free media (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; McCutchen et al., 2002; Pozzo-Miller, 2006; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). When using serum-free media, equine serum was titrated down from 20% over 3 days (10% at 4 div; 5% at 5 div; serum-free at 6 div). Serum-free media consisted of Neurobasal-A plus B-27 supplement and L-glutamine (1mM). All tissue culture reagents were obtained from InVitrogen (Carlsbad, CA), except for glucose, which was obtained from Sigma (St. Louis, MO).

PARTICLE-MEDIATED GENE TRANSFER

After 7 days *in vitro*, slices were transfected as previously described (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b). Briefly, plasmid cDNA for enhanced yellow fluorescent protein (eYFP; Clontech; Mountain View, CA) was introduced by biolistic transfection using a Helios gene gun (Bio-Rad; Hercules, CA). Plasmid cDNA was precipitated onto 1.6 μ m-diameter colloidal gold at a ratio of 2 μ g DNA/1mg gold, and then coated onto Tefzel tubing. Slices on tissue culture inserts were bombarded with gold particles accelerated by ~100psi He from a distance of 15mm using a modified gene gun nozzle (Alonso et al., 2004). Prior to transfection, an antibiotic/antimycotic mixture (1:100; penicillin/streptomycin/amphotericin B) was added to culture media to prevent contamination during biolistic transfection. The antibiotic/antimycotic mixture was only used during biolistic transfection and was removed after 24hrs to avoid the consequences of network desinhibition from their known actions on GABA_A receptor channels (Twyman et al., 1992).

TREATMENT CONDITIONS

Slices were randomly assigned to the following groups: (1) serum-containing media controls; (2) slices kept in serum-free media; (3) BDNF (250ng/mL; provided by Amgen; Thousand Oaks, CA) in the presence of serum; (4) BDNF (250ng/mL) in serum-free media. A droplet (50 μ L) of medium was gently applied onto each slice to facilitate penetration, followed by full medium exchange (1mL per tissue culture well). All treatments lasted 48hrs, beginning 48hrs after biolistic transfection, and slices were coded for subsequent “blind” quantitative analyses of dendritic spine density and morphology by an investigator unaware of treatment groups.

LASER SCANNING CONFOCAL MICROSCOPY

After 48hrs in each of the treatment conditions, slices were fixed by immersion in 4% paraformaldehyde in 100mM phosphate buffer (overnight at 4°C), and washed in phosphate buffer saline (PBS). Filter membranes around each slice were trimmed, and each slice was individually mounted on glass slides and coverslipped using Vectashield (Vector Laboratories; Burlingame, CA). Transfected pyramidal neurons located in the CA1 region displaying eYFP fluorescence throughout the entire dendritic tree and lacking signs of degeneration (e.g. dendritic blebbing) were selected for confocal imaging. High-resolution images of secondary and tertiary branches of apical dendrites were acquired with a Fluoview FV-300 laser scanning confocal microscope (Olympus; Center Valley, PA) using an oil immersion 100X (NA 1.4) objective lens (PlanApo). eYFP was excited using the 488nm line of an Argon laser, and detected using standard FITC filters. Series of optical sections in the z-axis were acquired at 0.1 μ m intervals through each dendritic branch. Figure 1A shows a representative slice culture

immunostained with anti-NeuN antibodies (Chemicon; Temecula, CA) to reveal the characteristic hippocampal organization (red; see below), and one transfected CA1 pyramidal neuron expressing eYFP (green).

ANALYSIS OF SPINE DENSITY

Dendritic spines of CA1 pyramidal neurons were identified as small protrusions that extended $\leq 3\mu\text{m}$ from the parent dendrite, and counted offline in maximum-intensity projections of the z-stacks using ImageJ software (National Institutes of Health). Protrusions longer than $3\mu\text{m}$ were rarely observed in CA1 pyramidal neurons in slice cultures at this developmental age (P7-P10 harvesting, 11 days *in vitro*) (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; De Simoni et al., 2003; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001), and since likely represent dendritic filopodia, they were not considered in the following analyses. Care was taken to ensure that each spine was counted only once by following its projection course through the stack of z-sections. Spines were counted only if they appeared continuous with the parent dendrite. Spine density was calculated by quantifying the number of spines per dendritic segment, and normalized to $10\mu\text{m}$ of dendrite length. Microscope calibrations were performed using $1.07\mu\text{m}$ fluorescent microspheres (Molecular Probes; Eugene, OR), which yielded a lateral resolution of 10.8 pixels per μm (i.e. 92nm per pixel).

MEASUREMENTS OF SPINE DIMENSIONS FOR SPINE TYPE CLASSIFICATION

The categorization of different morphological spine types was performed as described (Tyler and Pozzo-Miller, 2003) (Fig. 1B). Briefly, spines were classified into three classical subjective categories (Peters and Kaiserman-Abramof, 1970; Peters and Kaiserman-Abramof, 1969), but based on objective geometric measurements of their dimensions. Spines were classified as stubby (type-I), mushroom (type-II) or thin (type-III) types based on the L/N and H/N ratios, where L is spine length, H is the maximum head width, and N is the maximum neck width (Harris et al., 1992; Koh et al., 2002). Following these criteria, stubby spines have a length that is similar to the diameter of the neck and is similar to the diameter of the head ($L \approx N \approx H$); mushroom spines have a greater H/N ratio ($H > N$); and the length of thin spines is much greater than their neck diameters ($L \gg N$) (Fig. 1B). The majority (~65%) of these dendritic spines have presynaptic partners as assessed by synaptobrevin staining (Tyler and Pozzo-Miller, 2001), and despite morphological differences, all the three spine types make synaptic contacts *in vitro* (De Simoni and Edwards, 2006). Spine dimensions were measured in maximum-intensity projections of the z-stacks using Image J by an investigator unaware of treatment groups. Table 1 presents the results of the quantitative analyses of spine density and morphology. Table 2 shows the number of slices, neurons and spines counted and measured in each treatment group, as well as the total dendritic length analyzed.

WESTERN BLOT ASSAYS

For immunoblots, 18 slices per experimental condition cultured for 11 div were homogenized in ice-cold RIPA buffer. Samples of $15\mu\text{g}$ of protein from whole homogenates were subjected to SDS-PAGE (4–20% gels, Bio-Rad) and transferred to Immobilon PVDF Transfer Membranes (Millipore). Immunoblots were performed with rabbit anti-TrkB (Calbiochem) and rabbit polyclonal antisera directed against the cytoplasmic domain of p75NTR (#9992, kindly provided by M. Chao; NYU). Detection of antibodies was conducted with goat anti-rabbit HRP conjugate (Bio-Rad). In order to normalize the expression of proteins between experimental conditions, an antibody directed against actin was used as loading control. The anti-actin antibody (#JLA20), developed by J.J.-C. Lin, was obtained from the Developmental Studies Hybridoma Bank under the auspices of NIH-NICHD and maintained by the Department of Biological Sciences, University of Iowa. Gels were scanned at 300dpi and the optical density of the bands was quantified using Image J.

IMMUNOCYTOCHEMISTRY

Slice cultures (11–12 div) were fixed by immersion in 4% paraformaldehyde in 100mM phosphate buffer (overnight at 4°C) and washed in PBS. Free-floating slices still attached to the support filter membrane were permeabilized and blocked with 10% goat serum, 2% bovine serum albumin and 0.25% Triton-100 in PBS overnight at 4°C. Slices were then incubated overnight (4°C) in primary anti-TrkB (Calbiochem) or anti-p75NTR (Chemicon) antibodies. Slices were then washed in PBS and incubated overnight (4°C) in FITC-conjugated secondary antibodies (Santa Cruz; Santa Cruz, CA). After rinsing in PBS, the procedure was repeated with a primary anti-PSD-95 antibody (Santa Cruz), followed by a secondary Texas Red-conjugated antibody (Santa Cruz). Finally, slices were mounted with Vectashield (Vector Laboratories). Images were acquired in an Olympus IX70 epifluorescence microscope using a Retiga-1300 cooled CCD monochromatic camera (Q-Imaging; British Columbia, Canada). Images were deconvolved using IPLab Spectrum (Scanalytics; Rockville, MD).

STATISTICAL ANALYSES

Data were analyzed statistically using unpaired Student's *t* test using Prism (GraphPad; San Diego, CA). $P < 0.05$ was considered significant. Data are presented as mean $\pm$ standard error of the mean (SEM).

RESULTS

BDNF INCREASES DENDRITIC SPINE DENSITY REGARDLESS OF THE PRESENCE OF EQUINE SERUM IN CULTURE MEDIA

We previously showed that BDNF increases the density of dendritic spines in secondary and tertiary branches of apical dendrites of CA1 pyramidal neurons either loaded with Alexa-594 during whole-cell recording (Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001) or biolistically transfected with eYFP (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b). In those studies, slice cultures were maintained in serum-free media during the BDNF exposure to avoid potential confounding effects of unknown factors in equine serum. We show here that the gradual removal of serum over a 3-day period starting at 4 div, and the maintenance of slices in fully defined culture media (Neurobasal-A plus B27 supplement) did not affect dendritic spine density in secondary and tertiary branches of apical dendrites of CA1 pyramidal neurons at 9–11 div (serum-free [SF] = 9.12 ± 0.64 spines per $10\mu\text{m}$ of dendritic length, 12 cells from 7 slices, *vs.* serum-containing medium [SM] = 7.60 ± 0.57 spines/ $10\mu\text{m}$, 13 cells from 9 slices; $p = 0.09$; Table 1 and Fig. 2A, B).

Since BDNF modulated the proportion of the three major morphological types of dendritic spines (Tyler and Pozzo-Miller, 2003), we compared the proportions of spine types between CA1 neurons maintained in the presence or absence of equine serum. Following well-accepted criteria based on their geometric dimensions, dendritic spines were classified as stubby (type-I), mushroom (type-II) and thin (type-III) spines (Harris et al., 1992; Koh et al., 2002) (Fig. 1B, see Methods for details). Indeed, CA1 pyramidal neurons maintained in serum-containing media had a higher proportion of stubby spines (SM = 0.6 ± 0.03 , 13 cells from 9 slices, *vs.* SF = 0.44 ± 0.02 12 cells from 7 slices; $p = 0.0004$) and a lower fraction of thin spines (SM = 0.1 ± 0.02 , *vs.* SF = 0.22 ± 0.02 ; $p < 0.0001$), while mushroom spines were unaffected by serum withdrawal (SM = 0.3 ± 0.02 , *vs.* SF = 0.34 ± 0.01 ; $p = 0.2208$) (Table 1, and Fig 2C). Thus, serum withdrawal causes an increase in the fraction of “immature” thin spines, while decreasing the proportion of “mature” stubby spines.

Considering the effect of serum withdrawal on the proportions of morphological types of spines, we revisited the effects of BDNF on CA1 pyramidal neurons maintained in the absence or presence of equine serum. Consistent with our previous observations (Alonso et al., 2004;

Amaral and Pozzo-Miller, 2007b; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001), application of BDNF (250ng/mL, 48hrs) to slices maintained in serum-free media increased dendritic spine density (SF = 9.12 ± 0.64 spines/ $10\mu\text{m}$ vs. SF+BDNF = 12.05 ± 1.32 spines/ $10\mu\text{m}$, 6 cells from 5 slices; $p < 0.05$; Fig. 2A, B). Then, we tested the effect of BDNF to slices maintained in culture media containing 20% equine serum, the standard protocol (Gahwiler, 1981; Pozzo Miller et al., 1993; Stoppini et al., 1991; Yamamoto et al., 1989). In the presence of serum, BDNF also increased spine density (SM = 7.60 ± 0.57 spines/ $10\mu\text{m}$ vs. 15 SM+BDNF = 9.39 ± 0.56 spines/ $10\mu\text{m}$, 10 cells from 7 slices; $p < 0.05$; Fig. 2D, E).

THE EFFECT OF BDNF ON DENDRITIC SPINE MORPHOLOGY DEPENDS ON THE PRESENCE OF EQUINE SERUM IN CULTURE MEDIA

We previously showed that BDNF specifically increases the proportion of stubby (type-I) spines in CA1 pyramidal neurons of slices maintained in serum-free media (Tyler and Pozzo-Miller, 2003). As we did for spine density, we also revisited the effects of BDNF on spine morphology comparing slices exposed in serum-free vs. serum-containing media. Consistent with our previous observations, application of BDNF (250ng/mL, 48hrs) to slices maintained in serum-free medium increased the proportion of stubby spines (SF = 0.44 ± 0.02 , 12 cells from 7 slices vs. SF+BDNF = 0.55 ± 0.05 , 6 cells from 5 slices; $p < 0.05$; Fig. 2C). In contrast, application of BDNF to slices maintained in serum-containing media decreased the proportion of stubby spines (SM = 0.60 ± 0.03 , 13 cells from 9 slices vs. SM+BDNF = 0.46 ± 0.01 , 10 cells from 7 slices; $p < 0.01$; Fig. 2F). In addition, BDNF increased the proportion of mushroom spines (SM = 0.30 ± 0.02 vs. SM+BDNF = 0.37 ± 0.01 ; $p < 0.05$), as well as thin spines (SM = 0.10 ± 0.02 vs. SM+BDNF = 0.17 ± 0.02 ; $p < 0.05$) when applied to slices maintained in serum-containing media (Fig. 2F). This effect of BDNF in the proportion of spine types is opposite to that observed in serum-free conditions, where the proportions of mushroom and thin spines, though not significant, show a trend towards a reduction in their proportions (Fig. 2C).

Since BDNF can activate both p75NTR and TrkB receptors, we next determined whether CA1 pyramidal neurons in slice cultures express these neurotrophin receptor types. Indeed, apical dendrites of CA1 pyramidal neurons display p75NTR and TrkB immunopositive puncta that colocalized with the postsynaptic protein, PSD-95 (Fig. 3A). Then, we tested whether the presence of equine serum in culture media affects the expression levels of p75NTR and TrkB receptors. Optical density (OD) analyses of Western immunoblots show that slices maintained in serum-free media express lower levels of both p75NTR and TrkB receptors compared to slices grown in serum-containing media (p75NTR 0.61 vs. 2.44 ODU normalized to actin; TrkB 0.03 vs. 0.59 ODU). In addition, the ratio of p75NTR-to-TrkB expression levels is higher in serum-free slices (16.68 p75NTR-to-TrkB ratio of OD units) compared to slices maintained in serum-containing media (4.11 ratio of OD units), even after BDNF exposure (SF +BDNF=11.57 vs. SM+BDNF=0.38) (Fig. 3B). These results suggest that p75NTR signaling may always predominate over TrkB, but at a lesser degree in slices maintained in serum-containing media. Taken together, these results suggest that the effects of BDNF on spine morphology are opposite depending on the presence or absence of equine serum in culture media, despite being able to increase spine density in both conditions.

DISCUSSION

Here, we have presented several novel observations regarding the role of equine serum present in culture media and the actions of BDNF on dendritic spine density and morphology in CA1 pyramidal neurons. First, we show that serum withdrawal did not affect spine density, but it increased the proportion of thin spines while decreasing the proportion of stubby spines. Second, serum withdrawal did not prevent the spinogenic action of BDNF (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-

Miller, 2001); however, BDNF had the opposite effect on the proportion of spine morphological types in the presence of serum-containing media, i.e. it increased the proportion of thin and mushroom spines while decreasing the proportion of stubby spines. Arguably, these three categories are snapshots of a continuous distribution of all possible spine morphologies, but they have been consistently correlated with neurological conditions (e.g. mental retardation) and behavioral states (e.g. learning), prompting a model in which they participate differentially in synaptic function and plasticity models of learning and memory (Bourne and Harris, 2007; Kasai et al., 2003). Third, slices maintained in serum-containing media show a lower p75NTR-to-TrkB expression level than serum-free slices, as determined by Western immunoblotting even after BDNF exposure, suggesting that the relative expression levels of BDNF receptors determines the outcome of its actions in terms of dendritic spine density and form in hippocampal pyramidal neurons. Taken together, these differential BDNF actions on dendritic spine morphology reveal an unexpected level of complexity in the consequences of BDNF signaling on dendritic morphology.

Our observation that the presence of equine serum in culture media affects the cellular response to BDNF raises caution when designing and performing experiments in cultured cells and tissue explants. This is particularly important when using organotypic slice cultures due to the high concentration of equine serum (20–25%) used in the standard protocol (Gahwiler, 1981; Pozzo Miller et al., 1993; Stoppini et al., 1991; Yamamoto et al., 1989). To avoid potential confounding effects due to unknown factors in the equine serum used in standard organotypic slice culture protocols, our prior studies used slices maintained in defined serum-free media, which was titrated down from 20% over the first 3 days in culture (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007b; McCutchen et al., 2002; Pozzo-Miller, 2006; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). Despite the widespread use of cultured preparations that include undefined sera in their medium formulations, very few reports describe differential effects due to serum presence beyond neuronal survival and/or differentiation (e.g. Takeshima et al. 1994). For example, serum starvation caused a translocation of Ca^{2+} -activated K^{+} channels to the plasma membrane without affecting total protein levels (Weaver et al. 2004).

BDNF has recently emerged as a powerful modulator of hippocampal synaptic function. BDNF release occurs in response to stimulus patterns associated with synaptic plasticity (Aicardi et al., 2004; Balkowiec and Katz, 2002; Gartner and Staiger, 2002), which strongly supports the role of this prominent neurotrophin in the activity-dependent functional and structural plasticity of excitatory glutamatergic synapses (Bramham and Messaoudi, 2005; Segal, 2005). Consistent with prior reports, we show here that BDNF increases dendritic spine density in hippocampal pyramidal neurons (Ji et al., 2005; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). The mechanism by which BDNF exerts this structural effect are beginning to be clarified: Ca^{2+} mobilization from IP_3 -sensitive intracellular stores followed by TRPC3-mediated membrane depolarization and Ca^{2+} entry (Amaral and Pozzo-Miller, 2007a, b), as well as activation of ERK1/2 (Alonso et al., 2004) and cAMP (Ji et al., 2005) signaling are necessary for BDNF's effects on dendritic spine density. However, the relative contribution of each BDNF receptor type, p75NTR and TrkB, to the modulation of spine density and structure remains unknown, unlike their opposing roles in hippocampal synaptic plasticity (Minichiello et al., 2002; Minichiello et al., 1999; Pang et al., 2004; Rosch et al., 2005; Woo et al., 2005; Xu et al., 2000), which prompted a model of opposing neurotrophin signaling through p75NTR and Trk receptors (Hempstead, 2006; Lu et al., 2005). Further studies are underway to unravel the role of each BDNF receptor to dendritic spine density and morphology.

Does BDNF-induced structural plasticity of dendritic spines have functional implications? At least for dendritic Ca^{2+} signals evoked by coincident pre and postsynaptic activity, the shift

towards a higher proportion of stubby spines after BDNF exposure seems to have significant consequences. Dendritic Ca^{2+} elevations evoked by coincident excitatory postsynaptic potentials (EPSPs) and back-propagating action potentials (bAPs) were larger than those triggered by bAPs alone, but only in CA1 neurons exposed to BDNF and not in control cells (Pozzo-Miller, 2006). Since BDNF increases spine density and modifies spine morphology, the aforementioned results may reflect a physiological consequence of the BDNF-initiated structural remodeling of dendritic spines. Along with several other physiological consequences of BDNF signaling in the hippocampus, i.e. modulation of presynaptic function (Tyler et al., 2002b), BDNF-induced dendritic remodeling likely contributes to its role in the establishment and activity-dependent refinement of neuronal networks necessary and fundamental for synaptic plasticity and hippocampal-dependent learning and memory (Amaral et al., 2007; Amaral and Pozzo-Miller, 2005; Bramham and Messaoudi, 2005; Chapleau and Pozzo-Miller, 2007; Tyler et al., 2002a).

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Figure 1. EXPERIMENTAL DESIGN

Hippocampal slice cultures were transfected with eYFP using a gene-gun and processed for laser-scanning confocal microscopy. Quantitative analyses of dendritic spine density and geometrical measurements of their dimensions for spine type categorization were performed in individually identified and selected CA1 pyramidal neurons. **A, left.** NeuN immunocytochemistry was performed to identify hippocampal cytoarchitecture and subregions (i.e. DG, dentate gyrus; CA3 and CA1). The white box shows the area CA1 used to sample eYFP-expressing pyramidal neurons. **middle.** Representative CA1 pyramidal neuron showing the apical dendritic tree used for quantitative analyses of dendritic spines. **right.** Apical dendritic segment representative of those selected for confocal imaging; individual dendritic spines are marked with arrows. **B,** Classification of spine morphological types is based on the spine length, the maximum diameter of the spine head, and the maximum width of the spine neck. For the purposes of this study, three different types of dendritic spines were defined:

stubby (type-I), mushroom (type-II) and thin (type-III) spines, to be consistent with our prior studies (see text for details).

Figure 2. BDNF INCREASES DENDRITIC SPINE DENSITY IN CA1 PYRAMIDAL NEURONS INDEPENDENTLY FROM THE PRESENCE OF EQUINE SERUM IN CULTURE MEDIA, BUT ITS EFFECT ON DENDRITIC SPINE MORPHOLOGY DEPENDS ON THE PRESENCE OF SERUM

A. Representative examples of dendritic segments of CA1 pyramidal neurons maintained in serum-free media (SF) and treated with 250ng/mL BDNF for 48hrs. In this and all subsequent figures, scale bar represents 2μm. B. Dendritic spine density expressed per 10μm of apical secondary or tertiary dendrite. C. Proportion of each morphological type of dendritic spine, expressed as a fraction of the total spine population. D. Representative examples of dendritic segments of CA1 pyramidal neurons maintained in serum-containing media (SM) and treated with 250ng/mL BDNF for 48hrs. E. Dendritic spine density expressed per 10μm of apical secondary or tertiary dendrite. F. Proportion of each morphological type of dendritic spine, expressed as a fraction of the total spine population. In this and all subsequent figures, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, after unpaired Student's *t* test (see text for details).

Figure 3. BOTH BDNF RECEPTORS p75^{NTR} AND TrkB ARE EXPRESSED IN CA1 APICAL DENDRITES OF POSTNATAL HIPPOCAMPAL SLICE CULTURES, BUT THEIR EXPRESSION LEVELS ARE DIFFERENTIALLY MODULATED BY THE PRESENCE OF EQUINE SERUM IN THE CULTURE MEDIA

A. Representative examples of immunostaining with anti-p75^{NTR} and anti-TrkB antibodies from the apical dendritic region of area CA1 of hippocampal slice cultures followed by image deconvolution microscopy (scale bar represents 10 μ m). The arrows in the left panels show co-localization with profiles immunolabeled with antibodies against the postsynaptic protein PSD-95, indicating a synaptic localization of both BDNF receptors. **B.** Representative examples of Western immunoblots of whole-tissue homogenates prepared from hippocampal slice cultures maintained in serum-free or serum-containing media using anti-p75^{NTR} and anti-TrkB antibodies. Gels were re-probed using antiactin antibodies as loading controls and for normalization of quantitative densitometric analyses.

TABLE 1
QUANTITATIVE RESULTS OF DENDRITIC SPINE ANALYSES

Treatment	Spine density (spines/10µm of dendrite)	Spine length (µm)	Spine head diameter (µm)	Spine neck width (µm)	Proportion of type-I (stubby)	Proportion of type-II (mushroom)	Proportion of type-III (thin)
Serum-free media (SF)	9.12±0.64	0.65±0.02	0.36±0.01	0.26±0.01	0.44±0.02	0.34 ± 0.01	0.22 ± 0.02
SF+BDNF	12.05±1.32 *	0.67±0.04	0.42±0.01 *	0.31±0.01 *	0.55±0.05 *	0.28±0.03	0.17±0.03
Serum-containing media (SM)	7.6±0.57	0.57±0.02	0.37±0.01	0.3±0.01	0.6±0.03	0.3±0.02	0.1±0.02
SM+BDNF	9.39±0.56 *	0.62±0.01	0.37±0.01	0.26±0.01 *	0.46±0.01 *	0.37±0.01 *	0.17±0.02 *

Quantitative results from the analyses of dendritic spine density, individual spine geometrical dimensions and proportions of spine types (with total number of spines as 1). Numbers are mean ± SEM. Asterisks denote significant statistical differences compared to SM controls at $p < 0.05$ after unpaired Student's t test (see text for details). The total number of CA1 pyramidal neurons and hippocampal slices, as well as the total length of dendrites and the total number of dendritic spines counted and individually measured used for these quantitative analyses is presented in Supplemental Table 1.

TABLE 2

TOTAL DENDRITIC LENGTH, CA1 PYRAMIDAL NEURONS, HIPPOCAMPAL SLICES AND INDIVIDUAL DENDRITIC SPINES SAMPLED FOR QUANTITATIVE ANALYSES

Treatment	Total length of dendrites (µm)	Number of neurons	Number of slices	Total number of spines
Serum-free media (SF)	2090.74	12	7	1952
SF + BDNF	1225.41	6	5	1450
Serum-containing media (SM)	1706.34	13	9	1309
SM + BDNF	2131.13	10	7	2056

Total number of different CA1 pyramidal neurons and hippocampal slices used for quantitative analyses of dendritic spine density and geometrical morphology. The total length of dendrites and the total number of dendritic spines counted and individually measured is presented to provide a sense of the statistical sample analyzed.