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BDNF Locally Potentiates GABAergic Presynaptic Machineries: Target-selective Circuit Inhibition

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Inhibitory neurotransmission is critical for neuronal circuit formation. To examine whether inhibitory neurotransmission receives target-selective modulation in the long term, we expressed the cDNA of brain-derived neurotrophic factor (BDNF), which has been shown to induce the augmentation of GABAergic synapses *in vivo* and *in vitro*, in a small population of cultured hippocampal neurons. At 48 h after transfection, the expression level of glutamic acid decarboxylase 65 (GAD65), a GABA synthetic enzyme that resides mainly in GABAergic terminals, was selectively enhanced around the BDNF-expressing neurons, in comparison with the neighboring control neurons interposed between the BDNF-expressing neurons and inhibitory neurons. Exogenous BDNF application for 48 h also increased the GAD level and enhanced the GABA release probability. These potentiating effects were attenuated in inhibitory synapses on neurons expressing a dominant negative form of the BDNF receptor (tTrkB). This suggests that postsynaptic BDNF-TrkB signaling contributes to the target-selective potentiation of inhibitory presynaptic machineries. Since BDNF is expressed in an activity-dependent manner *in vivo*, this selectivity may be one of the key mechanisms by which the independence of functional neuronal circuits is maintained.

Keywords: GABA, GAD, hippocampus, independence, inhibitory synapse, plasticity

Introduction

Inhibitory neurotransmission, which is primarily mediated by GABA in the central nervous system (CNS), is critical for neuronal excitability. Recent electrical recordings in behaving animals have highlighted the importance of inhibitory transmission in natural neuronal activity. It was found that in any given behavioral condition, >90% of hippocampal CA1 pyramidal neurons are considered to be silent, whereas almost all of the inhibitory interneurons are active (Freund and Buzsaki, 1996; Henze *et al*., 2000). These results suggest that the firings of excitatory principal neurons are strictly suppressed *in vivo*.

In most types of GABAergic neurons, a single inhibitory neuron usually has thousands of target excitatory neurons (Freund and Buzsaki, 1996). Therefore, if inhibitory transmission takes on a role in information processing that goes beyond simple inhibition, selective modulation of the different inhibitory synapses appears to be required. However, little is known about the target selectivity in inhibitory augmentation, especially with respect to the long-term effects.

Brain-derived neurotrophic factor (BDNF) is a homo-dimer of a 15 kDa secretory protein, which is predominantly expressed in the CNS (Lewin and Barde, 1996). This protein is produced primarily by excitatory neurons such as pyramidal and granule cells in the hippocampus (Ernfors *et al*., 1990). Recent experiments using genetically modified mice have suggested a number of functions of BDNF and its receptor, TrkB, after birth, including its involvement in memory and learning (Gorski *et al*., 2003; Linnarsson *et al*., 1997; Minichiello *et al*., 1999).

The expression of BDNF is regulated by neuronal activity (Castren *et al*., 1992; Tabuchi *et al*., 2000), and the expression level varies extensively among the same type of neurons (Tokuyama *et al*., 2000). The distribution patterns of BDNF and TrkB (Aoki *et al*., 2000; Klein *et al*., 1990; Marty *et al*., 1996) in neurons suggest that BDNF can behave both as an autocrine factor (Kokaia *et al*., 1993; Zakharenko *et al*., 2003) as well as a retrograde/anterograde trans-synaptic messenger (Drake *et al*., 1999; Gartner *et al*., 2000). Taken together, these findings suggest that BDNF acts locally around activated neurons.

The long-term effects of BDNF on the formation and functional activation of inhibitory GABAergic synapses have been well documented (Baldelli *et al*., 2002; Rutherford *et al*., 1997; Seil and Drake-Baumann, 2000; Vicario-Abejon *et al*., 1998). In particular, BDNF-induced maturation of GABAergic presynaptic functions has been demonstrated in many experimental systems, both *in vivo* and *in vitro* (Huang *et al*., 1999; Marty *et al*., 2000; Mizuno *et al*., 1994; Yamada *et al*., 2002). This was assessed by the expression level of glutamic acid decarboxylase (GAD), maximal GABA release and other methods. Thus, BDNF is a candidate molecule for mediating the selective enhancement of inhibitory inputs on some of the excitatory neurons expressing BDNF. To confirm this, we have developed methods for expressing BDNF and its dominant negative receptor in <1% of a population of neurons. We have also analyzed whether the changes in the properties of GABAergic synaptic machineries can distinguish the target.

Materials and Methods

Primary culture

Cultured hippocampal neurons were prepared as previously described (Shitaka *et al*., 1996), with some modifications. Briefly, whole brains were isolated from embryonic day 18 Wistar rats (SLC, Shizuoka, Japan), and the hippocampi were dissected out and treated with 0.25% trypsin (Difco Laboratories, Detroit, MI) and 0.01% deoxyribonuclease I (Sigma, St Louis, MO) at 37°C for 30 min. The cells were suspended in Neurobasal medium (Invitrogen, San Diego, CA) containing 10% fetal bovine serum (Sankojunyaku, Tokyo, Japan), and were plated at a density of 50 000 cells/cm^2 on polyethyleneimine (Sigma)-coated glass coverslips (Matunami Glass Ind. Ltd, Japan), each equipped with a flexiPERM (Sartorius, Göttingen, Germany) that created eight wells of 0.7 × 1.0 cm^2. Twenty-four hours after plating, the medium was changed to serum-free Neurobasal medium supplemented with 2% B27 (Invitrogen).

Transfection

Truncated TrkB (tTrkB, mouse trkB.t1 1–1428, which lacks the kinase domain), control β-galactosidase (both gifts from Dr Rita J. Balice-Gordon),

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and pre-pro BDNF (a gift from Dr Masami Kojima) were each subcloned into the pIRES-hrGFP expression vector (Stratagene, La Jolla, CA) that carries the CMV promoter upstream and an internal ribosomal entry site (IRES)-humanized *Renilla reniformis* green fluorescence protein (hrGFP) downstream. Each gene of interest and hrGFP are thus transcribed as a single mRNA and then translated into separate proteins. DNA purified on a QIA filter (Plasmid Midi Kit; QIAGEN, Hilden, Germany) was used to transfect cells. A calcium phosphate precipitation method was used for transfecting cells after 5 (for tTrkB) or 7 (for BDNF) days in culture.

We defined cDNA-expressing cells as those with green fluorescence intensity >50, in 8-bit images, taken with a 0.5 s exposure. Under these conditions, morphological changes in neurons expressing tTrkB or BDNF were not apparent, and there were no significant differences in the average numbers of primary neurites or soma sizes in comparison with control-transfected neurons (some of the data is presented in the Results section). The number of neurites passing through the circles was counted and used for quantification.

Cell Treatment

For all experiments except those involving BDNF expression, 10 ng/ml of recombinant human BDNF (a gift from Sumitomo Pharmaceuticals, Osaka, Japan.) was added to the culture medium for 1 h in the case of Fos induction or 48 h in the case of other experiments. The addition was made after 7 days of *in vitro* growth and 48 h after cDNA transfection. In the case of drug treatment, the following drugs were added 30 min prior to the treatment with BDNF: 50 μM picrotoxin (Sigma), 200 μM saclofen (Tocris, Bristol, UK), 2 μM tetrodotoxin (TTX; Wako Chemicals), 1 mM *N*-nitro-L-arginine methyl ester (L-NAME; Sigma), 3 μM AM281 (Tocris) or a mixture of 100 μM 2-amino-5-phosphonovaleric acid (APV; Sigma), 30 μM 6-cyano-7-nitroquinoxaline (CNQX; Sigma) and 150 μM (*R*,*S*)-α-methyl-4-carboxyphenyl-glycine (MCPG; Tocris). The effects of all the drugs other than MCPG and saclofen were confirmed electrophysiologically.

Immunocytochemistry

Cells were treated sequentially with 4% paraformaldehyde at 4°C for 30 min, 0.1% Triton X-100 for 15 min, 2% goat serum/PBS for 1 h, and then with primary antibody, overnight, at 4°C. The primary antibodies used were anti-GAD65 (mouse monoclonal GAD-6, 1:500 dilution; Developmental Studies Hybridoma Bank, University of Iowa, IA), anti-MAP2 (rabbit, 1:1000 dilution; Biogenesis, UK), anti-TrkB (rabbit, 1:300 dilution, recognizes the intracellular domain of gp145; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HA (mouse, 1:500 dilution; Roche Diagnostics Corp.), anti-dystrophin (mouse, DY2, a gift from Dr F. Saito at Teikyo University), anti-GABA$_A$ receptor β2/3 (mouse, 1:200 dilution; 62–3G1, Upstate Biotechnology, NY) and anti-Fos (rabbit, 1:300 dilution; Oncogene Research Products). After washing, the cells were incubated with a fluorochrome-conjugated secondary antibody (Alexa 350-, 568- or 594-conjugated anti-rabbit IgG; Alexa 350- or 594-conjugated anti-mouse IgG; all at 1:400 dilution; Molecular Probes, Eugene, OR) for 1 h at room temperature. The specificity of GAD-6 for GAD65 among mixtures of brain proteins has been well characterized (Chang and Gottlieb, 1988). The rabbit anti-MAP2 antibody appears to have cross-immunoreactivity in the nucleus (as shown in Fig. 1*A*, blue), but we confirmed that it could safely be used as a neuronal marker, using another mouse monoclonal antibody from Chemicon.

Fluorescent images were acquired with an Orca II cooled CCD camera (Hamamatsu Photonics, Shizuoka, Japan).

Quantitative Fluorescence Image Analysis

Eight-bit digitized images were taken with a fixed exposure time that was carefully determined so as not to include pixels saturated in intensity. A linear relationship of fluorescence with intensity in the images was confirmed in the range used. Images were analyzed using Scion Image software (Scion Corp., Frederick, MD) and Metamorph (Universal Imaging Co.). For all images, the backgrounds were automatically subtracted prior to analysis using the rolling ball method in Scion Image, but no other modifications were applied to the intensity values. To quantify the expression level of GAD65, three methods were applied as follows.

First, to control the analyses, we defined interposed neurons as those within rhombuses, composed of two regular triangles that connected the somas of BDNF-expressing neurons and inhibitory neurons as shown in Figure 1*A*. To compare the GAD level around BDNF-expressing neurons and interposed neurons, we used the average intensity of GAD staining in a circle of radius 17.5 μm around the center of the soma. This radius was determined as being approximately twice the average soma

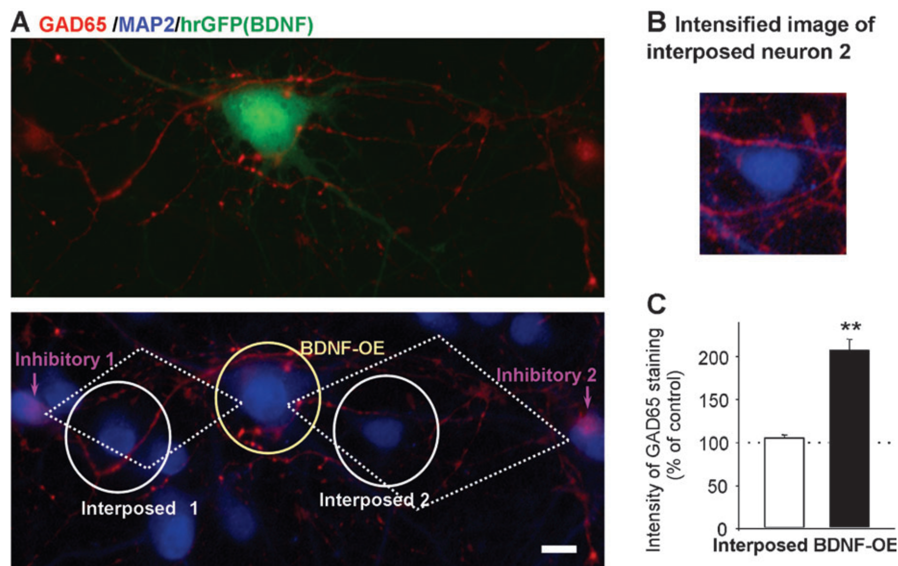

Figure 1. Increases in GAD65 signals around BDNF-overexpressing neurons. (*A*) Immunofluorescent signals for GAD65 (red, upper and lower panels) are clearly augmented around a BDNF-overexpressing neuron (BDNF-OE) visualized with coexpressed hrGFP (green, upper panel). Such signals are not apparent around neurons interposed between the BDNF-overexpressing neuron and inhibitory neurons. The interposed neurons used to gather the data shown in *C* are defined as MAP2-positive cells (blue, lower panel) whose soma are localized approximately within 60–120° rhombuses drawn between BDNF-overexpressing neurons and GAD65-positive cell bodies (presumably inhibitory neurons). The circles ($r = 17.5$ μm) represent the areas in which GAD65 was quantified for the data shown in *C*. Scale bar, 20 μm. (*B*) GAD65 puncta around an interposed neuron 1 in *A* become distinct when the red intensity in the image is artificially enhanced, suggesting that other synaptic contacts exist besides the intense puncta shown in *A*. (*C*) The average intensities of GAD65 immunofluorescence around BDNF-overexpressing neurons (BDNF-OE, $n = 120$) and interposed neurons ($n = 259$) show a clear difference (**$P < 0.01$, Student's *t*-test).

radius, and such circles included the largest soma and many of the GAD-puncta seen on thin neurites around the soma. To normalize for bias in each image, the values were divided by the average value obtained from four non-transfected neurons that happened to be present at the corners of the image. This method represented our best possible strategy for analyzing the terminals innervating neurons with no hrGFP, partly because we had previously confirmed that soma size and GAD level had no correlation (r^2 = 0.03 for BDNF neurons and 0.09 for interposed neurons).

Secondly, to determine the effects of bath-applied BDNF with or without drug treatments, the average overall fluorescence intensity (0–255) of 10 randomly selected fields ($10 \times 0.34 \times 0.34$ mm^2) in each well (as in Fig. 3*B*) was normalized with respect to the values of sister cultures that were not treated with BDNF. For each experiment, we obtained results from 6–9 wells, and each experiment was performed at least in triplicate.

Finally, during analyses on tTrkB-hrGFP- or control lacZ-hrGFP-expressing neurons, regions labeled with hrGFP (areas in images inside the cDNA-expressing neurons and very close surrounding regions) were clipped out, and the GAD image present inside was extracted. For the clipping, pixels with intensity over 50 in 8-bit hrGFP images (taken with an 8 s exposure) were assigned as 1, while the remainder were assigned as 0, and the resultant digitized images were then multiplied to the GAD65 image. The extracted GAD signals were quantified as the average intensity.

Nuclear Fos immunoreactivity was measured by drawing a circle of radius 2.5 μm at the center of the nuclei (Hoechst-stained areas). This radius was determined to be half of the average radius of the nucleus. It was large enough to obtain stable values for any part of the nucleus and still small enough to be included in the smallest nucleus.

Electrophysiology

Whole cell patch-clamp recordings were performed in transfected neurons as previously described (Bolton *et al.*, 2000). HrGFP-expressing cells were selected under an Olympus BX50WI fluorescence microscope. For recording $GABA_A$ receptor-mediated synaptic currents, we used an extracellular solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.4), 20 μM CNQX (Sigma) and 50 μM AP5 (Sigma), with or without 2 μM TTX, as well as an intracellular solution consisting of 140 mM CsCl, 0.2 mM $CaCl_2$, 2 mM EGTA, 4 mM MgATP, 5 mM QX314 and 5 mM HEPES (pH 7.2). Cells were held in a voltage clamp mode at a potential of −70 mV. Signals were recorded with an AXOPATCH 200B amplifier (Axon Instruments) with clamp-ex and clamp-fit, digitized by a DIGIDATA 1321A (Axon Instruments) at 10 kHz sampling frequency, filtered at 1 kHz and then analyzed with Igor software (Wave Metric Inc.).

Results

Selective Increases in GAD65 in Terminals on BDNF-overexpressing Neurons

BDNF has been shown to have highly reproducible effects, both *in vitro* and *in vivo*, on increasing the expression level of GAD, which is the rate-limiting enzyme in the synthesis of GABA (Huang *et al.*, 1999; Marty *et al.*, 2000; Yamada *et al.*, 2002). However, it is unclear whether this effect is selective among the target neurons. We analyzed this issue by detecting GAD65, a major subtype of GAD distributed predominantly in the presynaptic terminals of GABAergic synapses (Kaufman *et al.*, 1991). In order to develop a model for neurons that strongly express BDNF, we transfected hippocampal cells with BDNF cDNA. The rate of expression was intentionally lowered to <1% of the population of neurons in order to mimic the activity-dependent expression of BDNF *in vivo*. This was done because it has been reported that the increased expression of BDNF during learning has been detected in only 5% of the population of neurons in a part of the monkey cortex (Tokuyama *et al.*, 2000). As a result, 48 h after transfection, the immunocytochemical punctate stainings of GAD65 were selectively larger and brighter around the BDNF-overexpressing neurons (Fig. 1A, red). This enhancement was indistinct in the case of the other neighboring neurons. To evaluate the difference quantitatively, we defined control neurons as those neurons interposed between BDNF-overexpressing neurons and inhibitory neurons, within the same images. While evaluating the analytical methods, we found that although they were not observed in the original image (Fig. 1*A*), several GAD puncta were present around the interposed neurons when the GAD intensity in the image was artificially enhanced (Fig. 1*B*).

Thus, we decided to avoid methods that required setting a threshold value so as to ensure that the information from lower intensity staining was not neglected. Instead, the overall average intensity of GAD staining in an area was taken as an indicator of the amount of GAD in this study. GAD signals around neurons were enclosed by a circle of radius 17.5 μm, as shown in Figure 1*A*, and the average intensities inside the circle were normalized by the average value obtained from four non-transfected neurons at the corners of the image. As a result, the level of GAD staining around BDNF-overexpressing neurons was increased to 2.08 ± 0.13 (n = 120 neurons, P < 0.01), while that around the interposed neurons was 1.05 ± 0.04 (n = 259) (Fig. 1*C*). Another control for transfection was taken using neurons carrying a larger plasmid, which included lacZ in the same vector, and these neurons showed no increase in GAD staining (1.02 ± 0.11, n = 11). This effect of BDNF-expression was completely inhibited by an inhibitor of TrkB receptor kinase, K252a (0.99 ± 0.04 of control value, n = 51). This increase cannot be attributed to the number of major neurites, because the average values are not significantly changed. The values for primary neurites were 11 ± 0.7 and 9.9 ± 0.5, and those for secondary neurites were 3.6 ± 0.2 and 3.1 ± 0.7, for BDNF-overexpressing neurons and control neurons, respectively (n = 36). It is most probable that the level of GAD enzyme present in the presynaptic terminals innervating the BDNF-overexpressing neurons was increased.

The BDNF-induced Increase in Presynaptic GAD65 is Significantly Attenuated by the Expression of Kinase-defective TrkB in the Postsynaptic Neurons

The effect of BDNF on the GAD65 level was so local that there were two possible mechanisms: either BDNF acted directly on the GABAergic presynaptic terminals or it acted as an autocrine regulator of postsynaptic neurons, thereby activating GABAergic presynaptic terminals via another trans-synaptic retrograde signal.

To determine the contributions of pre- or postsynaptic neurons to the enhancement of GABAergic presynaptic terminals, we inhibited TrkB which is the BDNF receptor, in the neurons on just one side of the synapse, in the presence of BDNF. A truncated form of TrkB (tTrkB), which lacks the intracellular tyrosine kinase domain, has been shown to act as a dominant negative receptor for BDNF (Castren *et al.*, 1992; Gonzalez *et al.*, 1999; Haapasalo *et al.*, 2001). We transfected cells with the tTrkB cDNA tagged with HA-antigen, and also transfected β-galactosidase (lacZ) cDNA as a negative control for gene expression.

In this paper, the contribution of only postsynaptic excitatory neurons was examined, because, to our surprise, the transfection of inhibitory neurons was minimal. Even when the control vector was used, almost all (>95%) the cells expressing hrGFP were non-GABAergic neurons, defined as MAP2-positive

and somatic-GAD65-negative. This was not due to a difference in viability, since the ratio of the two types of neurons was not affected by the transfection (data not shown).

The tTrkB-expression was first determined immunocytochemically with an anti-HA antibody, 48 h after transfection. We confirmed that all the hrGFP-expressing neurons expressed HA (data not shown).

To confirm that the tTrkB-expressing neurons had lost their responsiveness to BDNF, we assayed the induction of nuclear Fos by BDNF using immunofluorescence, since the c-fos protein has been shown to be induced via the activation of TrkB in NIH-3T3 cells (McCarty and Feinstein, 1999). Exogenously applied BDNF did not increase the Fos expression in the tTrkB + hrGFP-expressing neurons (Fig. 2*B*), whereas it did increase the Fos levels in the control lacZ + hrGFP-expressing neurons (Fig. 2*A*, yellow signal in the nucleus in the merged image). These data (Fig. 2*C*) demonstrate that the tTrkB-expressing neurons were unresponsive to BDNF.

Since we applied BDNF in the medium at a 100-fold higher concentration than the K_d of Trk and neurotrophin, it is logical to suppose that the inhibition by tTrkB should be confined to the expressing neurons. Even pumping out of the excess ligand from the synaptic clefts appears to be impossible, partly because tTrkB is believed to be defective in the internalization with the ligand (Du *et al.*, 2000), and also because the synaptic clefts in cultured neurons are exposed to a volume of medium that is >1000-fold larger than the volume of the tTrkB-expressing neurons. In fact, we confirmed that the average Fos response of other cells in the same well as the tTrkB-expressing neurons, remained unaltered in this system (as shown in Fig. 2*B*, red, plus data not shown).

Thus, we confirmed the establishment of an experimental system in which only a small number of neurons lost their TrkB function, while most of the surrounding neurons showed normal responses to exogenously applied BDNF.

Next, we confirmed that treatment with 10 ng/ml BDNF increased the immunofluorescence signal of GAD65. This is in agreement with previously reported similar results of *in vivo*, slice and culture experiments (Huang *et al.*, 1999; Marty *et al.*, 2000; Yamada *et al.*, 2002). Regarding the time course, 6–12 h were required for significant changes to be observed and 48–72 h were necessary for stable changes (data not shown); thus, BDNF treatment was performed for 48 h. There was a 1.6-fold overall increase in GAD (Fig. 3, black column).

Next, we determined whether the effect of BDNF on GAD65 was mediated by neurotransmission. This is because BDNF has been shown to acutely alter neurotransmission by increasing neurotransmitter release (Zakharenko *et al.*, 2003), causing a form of long-term potentiation (Kang and Schuman, 1995) and decreasing inhibitory inputs (Tanaka *et al.*, 1997) at postsynaptic sites. As the results show, treatment of cells for 30 min prior to and during BDNF treatment with the following reagents did not have any significant effects on the BDNF-induced increases in the GAD65 level. The reagents used were TTX, which blocks action potentials; a mixture of glutamate receptor antagonists; picrotoxin, a $GABA_A$ antagonist; and saclofen, a $GABA_B$ antagonist that blocks long-term potentiation of GABAergic synapses (Komatsu, 1996) (Fig. 3).

We then assayed the GAD65 signals in the tTrkB-expressing neurons. In order to quantitatively analyze the GAD65 immunoreactive signals in the hrGFP-expressing neurons, GAD65 images inside the boundary line of hrGFP-fluorescence were clipped (Fig. 4*A*), and the overall GAD signals included in these clipped images were quantified (Fig. 4*B*). Treatment for 48 h with

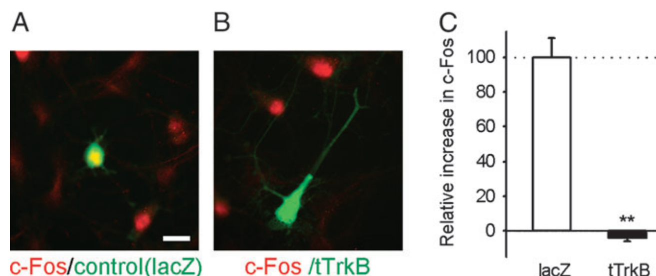

Figure 2. Confirmation of the lack of BDNF-induced response by nuclear Fos expression in neurons expressing tTrkB, a kinase-defective receptor for BDNF. (*A*, *B*) Treatment with 10 ng/ml BDNF for 1 h induces expression of nuclear Fos (red) in control lacZ + hrGFP-expressing neurons (*A*, yellow in the merged image), but not in tTrkB + hrGFP-expressing neurons (*B*). Scale bar, 20 μm. (*C*) Treatment with 10 ng/ml BDNF induces a significant increase in nuclear Fos expression in lacZ-expressing neurons, which is suppressed in tTrkB-expressing neurons (**$P < 0.01$ as compared with the lacZ-expressing neurons, Mann-Whitney *U*-test, $n = 116$–341 neurons). The increase in Fos fluorescent intensity in neurons expressing hrGFP is expressed as a percentage of the increase observed in the lacZ-expressing neurons.

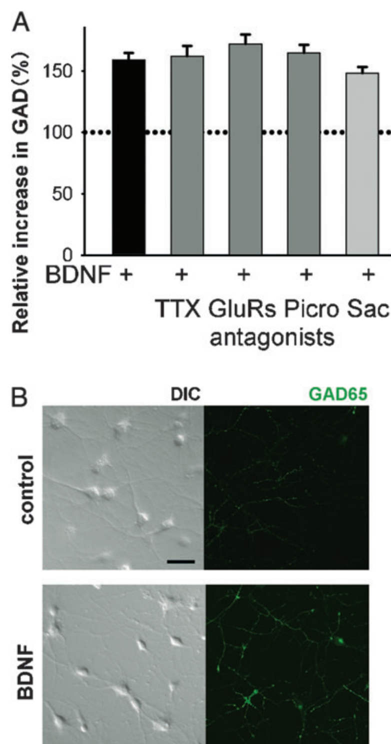

Figure 3. Exogenous BDNF increases the GAD65 level independently of neurotransmission. (*A*) The BDNF-induced increase in GAD65 immunofluorescence intensity was quantified and is shown as a ratio to the value from cultures without BDNF. The level was not influenced by treatment with: tetrodotoxin (TTX), which blocks action potentials; a mixture of the glutamate receptor antagonists AP5, CNQX and MCPG (GluRs antagonists); the $GABA_A$ antagonist picrotoxin (Picro); or the $GABA_B$ antagonist saclofen (Sac), each applied 30 min prior to the BDNF treatment. The ratios are: BDNF only, 1.6 ± 0.1 ($n = 17$ wells); + TTX, 1.6 ± 0.1 ($n = 8$); + GluRs antagonists, 1.7 ± 0.1 ($n = 7$); + Pic, 1.7 ± 0.1 ($n = 6$); and + Sac, 1.5 ± 0.1 ($n = 9$). There are no significant differences among these ratios. (*B*) DIC images and corresponding immunofluorescent images of GAD65 (green) on hippocampal neurons cultured in the absence or presence of 10 ng/ml BDNF for 48 h from 7 days *in vitro*. Scale bar, 40 μm.

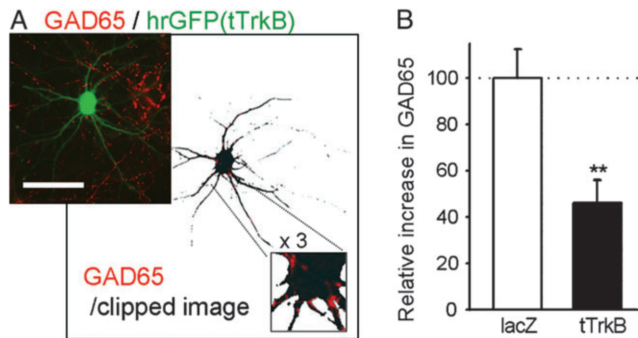

Figure 4. Contribution of BDNF-TrkB signaling in postsynaptic neurons to the increase in presynaptic GAD65. (*A*) Top left: A fluorescent image of GAD65 staining (red) and a tTrkB + hrGFP-expressing neuron (green). Scale bar, 40 μm. Middle: A clipped image of hrGFP (black) overlaid with that of GAD (red, intensified for clarity). Bottom right: A 3-fold magnification of part of the overlaid image. (*B*) Attenuation of the BDNF-induced increase in GAD65 signal on tTrkB + hrGFP-expressing neurons. The average GAD65 signal intensities within the clipped images were measured, and the increase induced by 10 ng/ml BDNF treatment for 48 h is shown as a ratio to that obtained from control lacZ + hrGFP-expressing neurons (**$P < 0.01$ compared with the control, Student's *t*-test, $n = 98$–175 neurons).

10 ng/ml BDNF increased GAD65 expression in lacZ-expressing neurons (Fig. 4, white column). In tTrkB-expressing neurons, however, this increase was significantly attenuated ($P < 0.01$ as compared with the lacZ-expressing neurons). This suggests that BDNF-TrkB signaling in postsynaptic neurons contributes to the BDNF-induced increase in GAD in presynaptic terminals.

These results showed that postsynaptic signaling affected a protein in the GABAergic presynaptic terminals, suggesting the involvement of a retrograde mediator from post- to presynaptic terminals and/or signaling via adhesion. The known retrograde mediators, NO and cannabinoid (Arancio *et al.*, 1996; Kreitzer *et al.*, 2002; Ohno-Shosaku *et al.*, 2002), would not be involved, because neither L-NAME, an inhibitor of NO synthesis, nor AM281, a cannabinoid receptor (CB1) antagonist, significantly attenuated the increases in GAD65 immunofluorescence intensity (ratio of cultures without BDNF: BDNF only, 1.6 ± 0.06; +L-NAME, 1.6 ± 0.01; and +AM281, 1.6 ± 0.01; $n = 7$ wells, respectively). The levels of two of the main postsynaptic machineries — dystrophin, a scaffold protein for mature GABAergic synapses (Brunig *et al.*, 2002), and extracellular $GABA_A$ receptor (detected without detergent treatment) (Brunig *et al.*, 2001) — were not significantly affected by exogenously applied BDNF (10 ng/ml, 96 ± 3.5% and 106 ± 2.8%; 50 ng/ml, 108 ± 3.5% and 104 ± 6.8% of control for dystrophin and $GABA_A$ receptor, respectively; $n = 7$ wells).

The BDNF-induced Enhancement of GABA Release Probability Was not Observed in tTrkB-expressing Neurons

To evaluate the effect of tTrkB expression on synaptic transmission, we recorded spontaneous and miniature GABAergic postsynaptic currents (sIPSCs and mIPSCs, respectively) in the cDNA-transfected excitatory neurons. Following 48 h of BDNF treatment, the average frequency of sIPSCs, which indicates GABA-release probability, was clearly increased in the control lacZ-expressing neurons, while this effect was suppressed in the tTrkB-expressing neurons (Table 1, Fig. 5*A*,*B*). These data for the control are consistent with those obtained from previous studies (Rutherford *et al.*, 1997, 1998). In this experiment, CNQX, AP5 and intracellular QX314 were used to block excitatory neurotransmission and recurrent transmission from the neurons being analyzed.

Table 1
BDNF increases the frequency of sIPSC in control lacZ-expressing neurons but not in tTrkB-expressing neurons

	lacZ	lacZ + BDNF	tTrkB	tTrkB + BDNF
Frequency of sIPSCs [Hz]	1.6 ± 0.22	3.5 ± 0.25*	2.6 ± 0.58	2.4 ± 0.37
Amplitude of sIPSCs [pA]	131 ± 14	137 ± 18	179 ± 23	133 ± 13
Frequency of mIPSCs [Hz]	0.62 ± 0.17	0.72 ± 0.26	1.0 ± 0.26	0.63 ± 0.15
Amplitude of mIPSCs [pA]	56 ± 3.9	58 ± 5.4	67 ± 7.5	58 ± 5.1

*$P < 0.05$ versus lacZ (sIPSCs: $n = 28$–32; mIPSCs: $n = 12$–18)

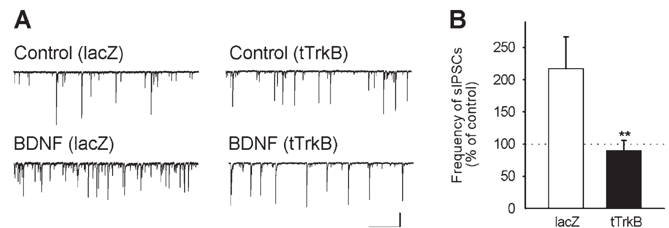

Figure 5. Contribution of BDNF-TrkB signaling in postsynaptic neurons to presynaptic change: BDNF increases GABA-release probability recorded in lacZ-expressing neurons but not in tTrkB-expressing neurons. (*A*) Traces of spontaneous GABAergic postsynaptic currents recorded from lacZ- or tTrkB-expressing neurons cultured in the presence or absence of 10 ng/ml BDNF for 48 h from 7 days *in vitro*. Scales, 100 pA, 5 s. (B) BDNF-induced increases in GABA-release probability to the transfected neurons shown as percentages of the frequency of control neurons (*$P < 0.05$ compared with the control lacZ-expressing neurons, Mann-Whitney *U*-test, $n = 31$–32 neurons).

Postsynaptic TrkB signaling in a neuron was probably required for the BDNF-induced increase in GABA-release probability to the neurons.

Discussion

We have shown here that GABAergic presynaptic machinery was potentiated in a target-selective manner, and we suggest that BDNF-TrkB signaling in postsynaptic neurons contributes significantly to the underlying mechanism.

We observed that the signal level of GAD was selectively enhanced around neurons overexpressing BDNF. This selectivity was confirmed by a quantitative analysis (Fig. 1*C*), even in comparison with neighboring neurons interposed between the BDNF-overexpressing neurons and inhibitory neurons, which probably receive GABAergic inputs from the same neurons.

This enhancement in the GAD signal probably reflects an increase in the protein level of the GAD enzyme within the presynaptic terminals, rather than synaptogenesis. This is because GAD-immunoreactive puncta, which probably represent presynaptic terminals, were found around almost all of the neurons but with less intensity, as shown in Figure 1*B*. Another possibility is that synaptogenesis is selectively enhanced in BDNF-treated neurons and that the newly generated synapses have intense GAD. However, this was not the case, at least for the similar GAD enhancement induced by exogenously applied BDNF (Fig. 3), because neither the frequency of the mIPSCs (Table 1) nor the levels of other synaptic proteins, dystrophin and extracellular $GABA_A$ receptor, were increased. Even if the

results are due to synaptogenesis, the validity of this type of modulation is not necessarily limited to cultures, because inhibitory synaptogenesis occurs in the adult brain in an activity-dependent manner (Zito and Svoboda, 2002). The most important finding presented here is that inhibitory potentiation like GAD increase can be found selectively in target neurons that are secreting BDNF.

Between the two possible underlying mechanisms of selectivity, our results support an initial effect of BDNF on postsynaptic neurons, which activates GABAergic presynaptic terminals via another trans-synaptic retrograde signal. This conclusion is drawn from the case of exogenous BDNF, in which the BDNF-induced increase in GAD signals was suppressed in terminals on the postsynaptic neurons deficient in BDNF-TrkB signaling.

In this study, a small but significant increase in GAD due to BDNF was also observed in data from tTrkB-expressing neurons at the same time as the attenuation. We believe that the main reason for this observation is the passing of enhanced signals from axons through the clipped images of the tTrkB-expressing neurons. BDNF has been shown to enhance the elongation and branching of axons of GABAergic neurons (Vicario-Abejon *et al.*, 1998), and is thought to directly affect the soma and dendrites of inhibitory neurons in a similar fashion (Fawcett *et al.*, 2000; Kohara *et al.*, 2003). The increase in tTrkB-expressing neurons may be due to these direct actions of BDNF on GABAergic neurons, since BDNF was applied in the medium, thus affecting all the neurons, including the inhibitory neurons.

Alternatively, the suppression by tTrkB might be partial. This is possible because there was an upward tendency in the amplitudes of mIPSCs and sIPSCs in the tTrkB-expressing neurons cultured without exogenous BDNF. This probably reflects an acute inhibitory effect of endogenous BDNF mediated by $GABA_A$ receptor phosphorylation (Jovanovic *et al.*, 2004) (Table 1). This tendency was not significant, but it suggests that exogenous BDNF partially acts in the longer term as compared with Fos induction.

Nevertheless, a significant suppression of BDNF action on GAD intensity and frequency of sIPSCs was found in tTrkB-expressing neurons. This supports the observation that BDNF acts via a mechanism involving postsynaptic TrkB signaling, resulting in these effects.

The underlying mechanism of this increase in the frequency of sIPSCs is hardly attributable to an increase in the firing rate of presynaptic GABAergic neurons, because the tTrkB neurons, which represent only one of many target neurons of GABAergic neurons, are few in number as compared with the other intact target neurons. Alternatively, a change in the failure rate of GABAergic synapses is probable. Failure is a property of synapses that miss transmitter release upon cell firing and is observed in many types of GABAergic synapses. For example, the connections between lacunosum-moleculare interneurons and pyramidal cells in the hippocampus exhibit a high failure rate ranging from 22 to 93% (Bertrand and Lacaille, 2001). Since this failure is a process that can be controlled at each presynaptic site, it is hypothesized that BDNF decreases the failure, and the sIPSC thus becomes more frequent. This effect would be influential in discriminating between one of several target neurons, and even between one of several synapses.

Our findings suggest that the frequency of GABAergic input onto a neuron can reflect, at least partially, the amount of BDNF expressed by the postsynaptic neuron. Since BDNF is expressed in an activity-dependent manner, this BDNF action would contribute to a method by which neurons in existing neural circuits avoid excess intervention by new inputs or new information (Abbott and Nelson, 2000; Hasselmo, 1994). This would be in addition to the homeostatic stabilization, which has been suggested earlier (Rutherford *et al.*, 1997, 1998).

Recently, GABAergic neurons in the hippocampus have been shown to fire synchronously in response to the rhythmic brain waves (theta waves) often observed in exploring animals (Klausberger *et al.*, 2003). It is likely that such synchronous oscillations of inhibitory inputs would delay or prevent the firings of neurons. In such environments, neurons that are innervated by more enforced inhibitory presynaptic terminals would remain silent as compared with other neurons, and would fail to reinforce their connections with excitatory inputs. Several hours after the repeated activation of a circuit, BDNF should be expressed by constituent neurons and should act to induce these neurons to receive such potentiated inhibitory inputs. This role of BDNF may have relation to a phenotype of mice which is deficient for the TrkB in the forebrain. This phenotype is the impairment in adaptation to new information (Vyssotski *et al.*, 2002).

In summary, we have shown that BDNF induces target-selective potentiation of GABAergic presynaptic terminals, an effect that at least partially requires BDNF-TrkB signaling in postsynaptic neurons. This action of BDNF may constitute a part of the activity-dependent plasticity, which maintains the independence of functional neuronal circuits.

Notes

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