Development/Plasticity/Repair

Endogenous Brain-Derived Neurotrophic Factor Triggers Fast Calcium Transients at Synapses in Developing Dendrites

Susanne B. Lang, Valentin Stein, Tobias Bonhoeffer, and Christian Lohmann

Max Planck Institute of Neurobiology, 82152 Martinsried-München, Germany

Brain-derived neurotrophic factor (BDNF) is involved in many aspects of the formation of functional neuronal networks. BDNF signaling regulates neuronal development not only globally, at the level of entire neurons or networks, but also at a subcellular level and with high temporal specificity; however, the spatiotemporal characteristics of intrinsic BDNF signaling are essentially unknown. Here, we used calcium imaging to directly observe intrinsic BDNF signaling in developing hippocampal neurons. We found that blocking intrinsic BDNF signaling with function-blocking BDNF antibodies (αBDNF) or K252-a reduced the frequency of spontaneously occurring fast and localized calcium rises in dendrites. Conversely, focal application of BDNF evoked fast and local dendritic calcium transients, which required activation of TrkB (tropomyosin-related kinase B) receptors as well as activation of voltage-gated sodium and calcium channels. Virus-mediated expression of PSD-95:CFP (postsynaptic density-95 tagged with cyan fluorescent protein) revealed that spontaneous local calcium transients occurred frequently at postsynaptic sites along the dendrite. The frequency of synaptically localized calcium transients was specifically reduced by blocking intrinsic BDNF signaling, whereas nonsynaptic calcium rises were not affected. Furthermore, focal BDNF delivery evoked localized and fast calcium elevations specifically at postsynaptic sites. Together, our results demonstrate that BDNF-dependent calcium signaling in developing hippocampal neurons is fast and occurs at synapses. These temporal and spatial characteristics of intrinsic BDNF signaling as well as its relative abundance renders BDNF an ideal signaling molecule in the establishment of specific synaptic connectivity and functional neuronal networks.

Key words: calcium signaling; development; BDNF; TrkB; postsynaptic site; hippocampus

Introduction

In the CNS, neurotrophins are well known for their ability to support neuronal survival and to control differentiation during development (Bibel and Barde, 2000; Huang and Reichardt, 2001). Besides these classical long-lasting effects, recent evidence has revealed acute effects of neurotrophins on neuronal plasticity. In particular, brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-related kinase B (TrkB) affect neuronal morphology in cortical circuitries through the control of dendritic and axonal branching in an activity-dependent manner (McAllister et al., 1995; Gorski et al., 2003) (for review see, McAllister et al., 1999). Moreover, BDNF signaling can modulate synaptic strength: it exerts acute effects on synaptic transmission (Lohof et al., 1993; Kang and Schuman, 1995; Levine et al., 1995, 1998) (for review, see Poo, 2001) and is involved in the induction and maintenance of hippocampal long-term potentiation (LTP) (Korte et al., 1995, 1996; Patterson et al., 1996; Kossel et al., 2001; Kovalchuk et al., 2002), as well as in the stabilization of synapses (Hu et al., 2005).

BDNF influences synaptic plasticity not only at a cellular but also at a subcellular level. For instance, apical and basal dendrites of the same pyramidal cells respond differently to the same neurotrophin (McAllister et al., 1995). Furthermore, BDNF release from a single cell has been shown to directly induce local dendritic growth and branching in nearby neurons in a distance-dependent manner within a relatively short time (Horch and Katz, 2002). In addition, it has been reported that local application of BDNF and simultaneous presynaptic activation mediate LTP in mature dentate granule cells (Kovalchuk et al., 2002). The expression and secretion of BDNF is known to be regulated by neuronal activity (Balkowiec and Katz, 2002; Gartner and Staiger, 2002), and BDNF release occurs at synapses, possibly at presynaptic or postsynaptic compartments or both (Hartmann et al., 2001; Kohara et al., 2001; Gartner et al., 2006) (for review, see Lessmann et al., 2003). These findings suggest that BDNF signaling is precisely regulated in space and time.

Calcium as an important second messenger acts downstream of BDNF and is thought to mediate some of its effects in a specific, fast and localized manner. For example, it has been shown that intracellular calcium signaling conveys the potentiating effect of BDNF at hippocampal synapses (Kang and Schuman, 2000; Kovalchuk et al., 2002). Moreover, the application of exogenous BDNF is known to induce a transient elevation in the intracellular calcium concentration, predominantly as a result of calcium release from internal stores (Berninger et al., 1993) but also dependent on calcium influx from the extracellular space (Marsh and Palfrey, 1996).

Although there is a substantial amount of knowledge about the cellular responses to exogenous BDNF, it has not yet been studied how endogenous BDNF signaling affects neuronal calcium responses. Therefore, we set out to observe directly intrinsic BDNF-induced calcium signaling in developing pyramidal neurons of hippocampal slice cultures. We asked whether spontaneous local calcium events are triggered by BDNF and investigated the mechanisms underlying BDNF-mediated calcium signaling. We found that intrinsic BDNF signaling elicited rapid and spatially restricted calcium rises in dendrites. BDNF-dependent local calcium transients required activation of TrkB receptors as well as voltage-sensitive sodium and calcium channels. Moreover, we observed that most of the BDNF-dependent spontaneous local calcium transients occurred at postsynaptic sites along the dendrite. Accordingly, focal BDNF delivery evoked localized calcium elevations specifically at single postsynaptic sites. Together, our data show that BDNF triggers fast and localized calcium transients in developing neurons, which preferentially occur at synapses.

Materials and Methods

Preparation of hippocampal slice culture. Hippocampal organotypic cultures from postnatal day 0 (P0) to P2 rats were prepared following the method of Stoppini et al. (1991). After decapitation, the hippocampi were dissected in ice-cold Gey's balanced salt solution, containing 0.5 ml of kynurenic acid and 0.5 ml of glucose, pH 7.2. Transversal slices were cut (400 μm) using a tissue chopper (McIlwain). Slices were plated onto Millicell membrane inserts (CM; Millipore, Billerica, MA) and incubated with DMEM medium containing 25% horse serum at 37°C, 7% CO_2 for up to 3 d.

Virus-mediated gene transfer. Postsynaptic density-95 tagged with cyan fluorescent protein (PSD-95:CFP) was expressed using viral infection. PSD-95:CFP (Graf et al., 2004) was cloned into the pSCA vector (DiCiommo and Bremner, 1998). Semliki forest viral particles were produced by transfecting pSCA–PSD-95:CFP and pHelper in a 1:2 ratio into human embryonic kidney HEK 293 cells. At 48 h after transfection, the supernatant was harvested, aliquoted, and stored at −80°C. The virus was activated by α-chymotrypsin treatment for 45 min and injected into the CA3 region.

Single-cell electroporation of CA3 pyramidal cells and bolus loading of the CA3 region. The recording chamber was temperature controlled at 35°C and perfused with HBSS consisting of the following (in mM): 14.61 $CaCl_2$, 4.93 $MgCl_2$, 4.07 $MgSO_4$, 53.33 KCl, 4.41 KH_2PO_4, 4.2 $NaHCO_3$, 1379.31 NaCl, 3.36 Na_2HPO_4, and 55.56 D-glucose. The high-affinity calcium indicator Oregon Green BAPTA-1 (250 μM, dissolved in distilled water, hexapotassium salt; Invitrogen, Carlsbad, CA) was electroporated into single neurons according to the method for dye electroporation (Haas et al., 2001; Rathenberg et al., 2003; Lang et al., 2006). Patch pipettes with a tip diameter of ∼1 μm and square voltage pulses (10 V, 20 ms duration) were used to fill individual neurons. For bolus loading (Stosiek et al., 2003), 50 μg (one vial) of Oregon Green 488 BAPTA-1 AM (Invitrogen) was dissolved in 5 μl of DMSO containing 10% of pluronic acid. After sonicating for 1 min, 50 μl of culture medium was added, and the solution was again sonicated. The tip of a patch pipette was filled with the dye solution, and the CA3 region of hippocampal slices on culture inserts was loaded by positioning the pipette tip in the stratum radiatum/stratum lacunosum-moleculare and applying short pressure pulses with a Picospritzer (General Valve, Fairfield, NJ). After a 1 h incubation, slices were used for experiments.

Imaging. Recordings were acquired using an Axioplan-2 microscope (Zeiss, Oberkochen, Germany) and a cooled CCD camera (VisiCam QE; Visitron Systems, Puchheim, Germany) controlled with MetaMorph software (Universal Imaging Corporation, West Chester, PA). Images were obtained at 0.3–10 Hz with either a Zeiss 0.95/63× or an Olympus Optical (Tokyo, Japan) 0.80/40× water immersion objective provided by H. Thoenen (Max Planck Institute of Neurobiology, Martinsried-München, Germany).

Application of BDNF and pharmacological agents. BDNF (26 μl, 15 μg/ml in PBS, containing 0.1% BSA to prevent binding to the storage and application ware; provided by H. Thoenen) was applied using a 100 μl pipette (final concentration of 200 ng/ml). Superfusion was interrupted during BDNF bath application; control experiments showed that interruption of superfusion alone did not affect calcium activity for the recording period (data not shown). Alternatively, BDNF was applied focally to the apical dendrites with pressure pulses through a micropipette using a Picospritzer (pulse duration of 40 ms). The BDNF application pipette (∼1 μm tip diameter) was placed at a distance of ∼10–20 μm from the dendrite, and three pulses were applied within 1 min. In control experiments, either PBS with 0.1% BSA or heat-inactivated BDNF (30 min at 95°C) was pressure applied to the dendrite.

The protein kinase antagonist K-252a (200 nM; Alexis Biochemicals, San Diego, CA) and the function-blocking mouse monoclonal antibody against BDNF (αBDNF) were bath applied (4 μg/ml, clone number 9, IgG) (Kolbeck et al., 1999; Kossel et al., 2001). For α-BDNF experiments, tubing was siliconized for 1 h, dried, and extensively washed to prevent adhesion of the antibody to tubing surfaces. Additionally, BSA (0.5 mg/ml) was added to the antibody solution. All other pharmacological agents were diluted in HBSS and bath applied: 1 μM tetrodotoxin (TTX), 5 μM cadmium chloride ($CdCl_2$), 20 μM cyclopiazonic acid (CPA), and 3 μM SKF 96365 hydrochloride [1–2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1 H-imidazole]. Tetanus toxin (TeTX) at 20 nM was added to the culture medium at least 12 h before the recordings.

Image analysis. Changes in $[Ca^{2+}]_i$ are derived from $\Delta F/F_0$, where F_0 is the baseline fluorescence, which is typically taken from the first 20 images of a recording. Global calcium transients were defined as fluorescence increases of at least 10% observed in all structures of the cell within the same focal plane. Locally occurring calcium transients were defined as local rises in fluorescence of >5% with a spatial extent of 1–30 μm along the dendrite. For analysis of the BDNF application experiments, changes caused by dendrite shifts attributable to the pressure pulse application were discarded. For statistical analysis, Wilcoxon's matched-pairs test was used.

Western blot. Hippocampal tissue from P3 rats was homogenized in gradient buffer (containing 0.32 M saccharose and 1 mM $MgCl_2$, pH7.4), 2× sample buffer (0.125 M Tris pH 6.8, 2% SDS, 20% glycerol, and 0.001% bromphenolblue), DDT, and a protease inhibitor cocktail (Sigma, Deisenhofen, Germany). HEK 293 cells served as controls. Aliquots of the lysates were then subjected to SDS-PAGE (6% acrylamide) and transferred to nitrocellulose membranes (transfer membranes; Millipore). The blots were incubated in blocking buffer of 0.05% Tween 20 and 0.5% gelatin in 10× Tris-buffered saline at 4°C overnight. After blocking, the nitrocellulose membrane was incubated with either a rabbit anti-TrkB antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit anti-actin (1:100; Sigma) for 1 h at room temperature. After incubation with the secondary antibody (1 h, room temperature, 1:10,000, peroxidase labeled anti-rabbit antibody), binding of the antibody was detected using an ECL detection kit (Amersham Biosciences, Braunschweig, Germany).

Immunohistochemistry. After viral infection with PSD-95:CFP, slices were fixed at 4°C overnight in paraformaldehyde (4% in 0.1 sodium phosphate buffer). After washing for 3 h in phosphate buffer (PB), slices were preincubated in a blocking solution (4% Triton X-100, 1.5% horse serum, and 0.1% BSA in PB, 4°C, overnight) and then incubated in the primary antibody [rabbit anti-synapsin (Chemicon, Temecula, CA), diluted 1:500 in 4% Triton X-100, 1.5% horse serum, and 0.1% BSA in PB] for 7–10 d. Slices were rinsed (3 h in PB) and then incubated for 2–3 d with the secondary antibody (anti-rabbit FITC, 1:50 in PB, 4°C). Stacks of high-resolution images (voxel size, x–y–z, 0.18 × 0.18 × 0.25 μm) from PSD-95:CFP-infected dendrites and anti-synapsin-labeled structures were obtained with a confocal microscope [Leica (Nussloch, Germany) SP2, sequential acquisition mode, 1.25/40× oil immersion objective]. Quantification of colocalization was performed using NIH ImageJ software. Puncta were considered colocalized when pixels of PSD-95: CFP puncta ("red" channel) and synapsin puncta ("green" channel) overlapped in high-magnification x–y and orthogonal (y–z) views ("yellow" pixels). The specificity of colocalization was tested using the reverse order of the synapsin stack rotated by 180°.
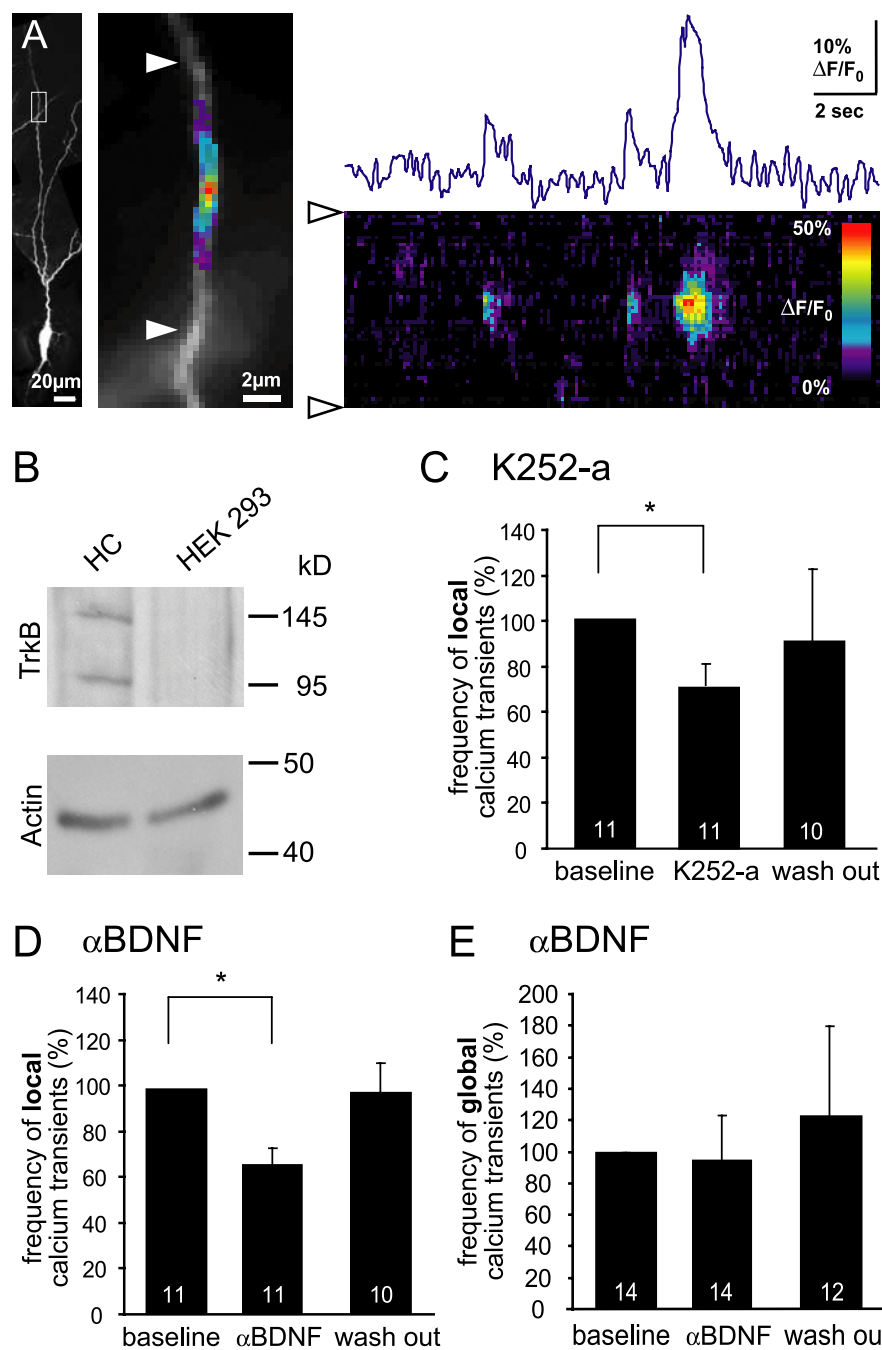
Figure 1. Frequency of spontaneously occurring local calcium transients in dendrites of developing hippocampal neurons is decreased by BDNF antibody or K252-a application. *A*, Electroporated single CA3 pyramidal neuron (P2 + 3 d *in vitro*) filled with Oregon Green BAPTA-1. Enlarged part of the dendrite shows spontaneous local increase in $[Ca^{2+}]_i$, represented as $\Delta F/F_0$ in pseudocolor. Arrowheads indicate the extent of the measured dendritic segment used for pseudo line scan and trace. *B*, Western blot analysis of TrkB receptor and actin in hippocampal lysates from developing rats (P2). Both full-length TrkB and truncated TrkB (molecular weights of 145 and 95 kDa, respectively) were present in the hippocampus (HC) but absent in the control (lysate of HEK 293 cells). Actin served as the loading control. *C*, Frequency of spontaneous local calcium transients before, during, and after bath application of K252-a normalized to baseline frequency. Calcium activity is significantly reduced during K252-a application. $n = 11$ neurons; error bars indicate SEM (*$p < 0.05$). *D*, Similarly, bath application of αBDNF causes a significant and reversible decrease in the frequency of spontaneous local calcium transients. $n = 11$ neurons (*$p < 0.05$). *E*, The frequency of spontaneous global calcium transients is unchanged during BDNF antibody application.

Results

Endogenous BDNF induces spontaneous local calcium transients

In our study, we focused on the first postnatal week when dendrites and axons undergo dynamic structural remodeling and form new synapses *in vivo* and in organotypic slice cultures (Dailey and Smith, 1996; Fiala et al., 1998). We observed spontaneous local rises of $[Ca^{2+}]_i$ in CA3 pyramidal neurons, which were labeled by single-cell electroporation (Fig. 1*A*). These local calcium transients occurred at a mean frequency of 73.8 ± 10.8 per minute and millimeter of dendrite. The average duration of such calcium rises was 680 ± 120 ms, and they were restricted to small stretches of 9.0 ± 0.6 μm of the dendrite. The amplitude of the transients (measured as $\Delta F/F_0$) was $17.3 \pm 0.6\%$, and their rise time was zero to four frames of 100 ms (median of two frames). Besides local calcium transients, hippocampal neurons also generated global calcium transients occurring in the soma, dendrite, and axon, presumably representing network activity (Garaschuk et al., 1998).

We investigated the role of endogenous BDNF for spontaneous local calcium signaling by interfering with the BDNF–TrkB signaling pathway. After confirming the presence of TrkB, the principal receptor for BDNF, in the developing hippocampus by Western blot analysis (Fig. 1*B*), we used either K252-a, which blocks tyrosine phosphorylation of Trk receptors, including TrkB, or functionblocking BDNF antibodies to inactivate endogenous BDNF. During bath application of 200 nM K252-a, we observed a significant and reversible reduction in the frequency of spontaneous local calcium transients ($-26.2 \pm 9.4\%$; $n = 11$; $p < 0.05$) (Fig. 1*C*). Temporal and spatial characteristics of local calcium transients were not changed. The frequency of global calcium transients remained stable. Similarly, bath application of BDNF antibodies (4 μg/ml) diminished the frequency of spontaneously occurring local calcium transients significantly ($-33.8 \pm 7.4\%$; $n = 11$; $p < 0.05$) (Fig. 1*D*). In contrast, the frequency of the global signals did not change significantly ($-5.4 \pm 28.5\%$) (Fig. 1*E*). These results show that interfering with the BDNF–TrkB signaling pathway reduces the frequency of spontaneous local calcium signaling, indicating that endogenous BDNF generates intracellular calcium signals via TrkB receptor activation.

Exogenous BDNF strongly increases the frequency of calcium transients

The observation of a reduced frequency of local calcium transients during inhibition of the BDNF–TrkB signaling pathway led us to ask whether additional exogenous BDNF would enhance calcium activity. We therefore applied exogenous BDNF (final concentration, 200 ng/ml) into the bath solution of the recording

chamber and observed a strong and reversible increase in the frequency of global calcium transients (+239.4 ± 88.9%; $n = 8$; $p < 0.05$) (Fig. 2A,B). In contrast to global calcium rises, the frequency of local calcium transients did not increase significantly during exogenous BDNF application (+41.9 ± 19.9%; $n = 12$) (Fig. 2C).

The increase in global activity might be a direct cellular response to BDNF. Alternatively, BDNF may modulate the network activity of the hippocampal circuit, resulting in an increase in global calcium activity (Sakai et al., 1997). To test whether the strong increase in the frequency of global calcium transients was a direct effect or caused by enhanced network activity, we used bolus loading (Stosiek et al., 2003) of Oregon Green BAPTA-1 AM to visualize network activity in CA3 pyramidal neurons. After fast BDNF application, we observed a sustained increase in the frequency of calcium transients in many neurons (before, 0.8 ± 0.3; after, 4.9 ± 0.8 global transients/min; $p < 0.05$; $n = 8$ slices; 80 cells) (Fig. 2D,F). The calcium transients were highly correlated across neurons, indicating that they reflected an increase in network activity. Averaging the responses from the entire set of cells showed that there was also an immediate but transient calcium response on BDNF application (Fig. 2G). When the experiments were repeated in slices preincubated (12 h) in TeTX to block network activity, BDNF
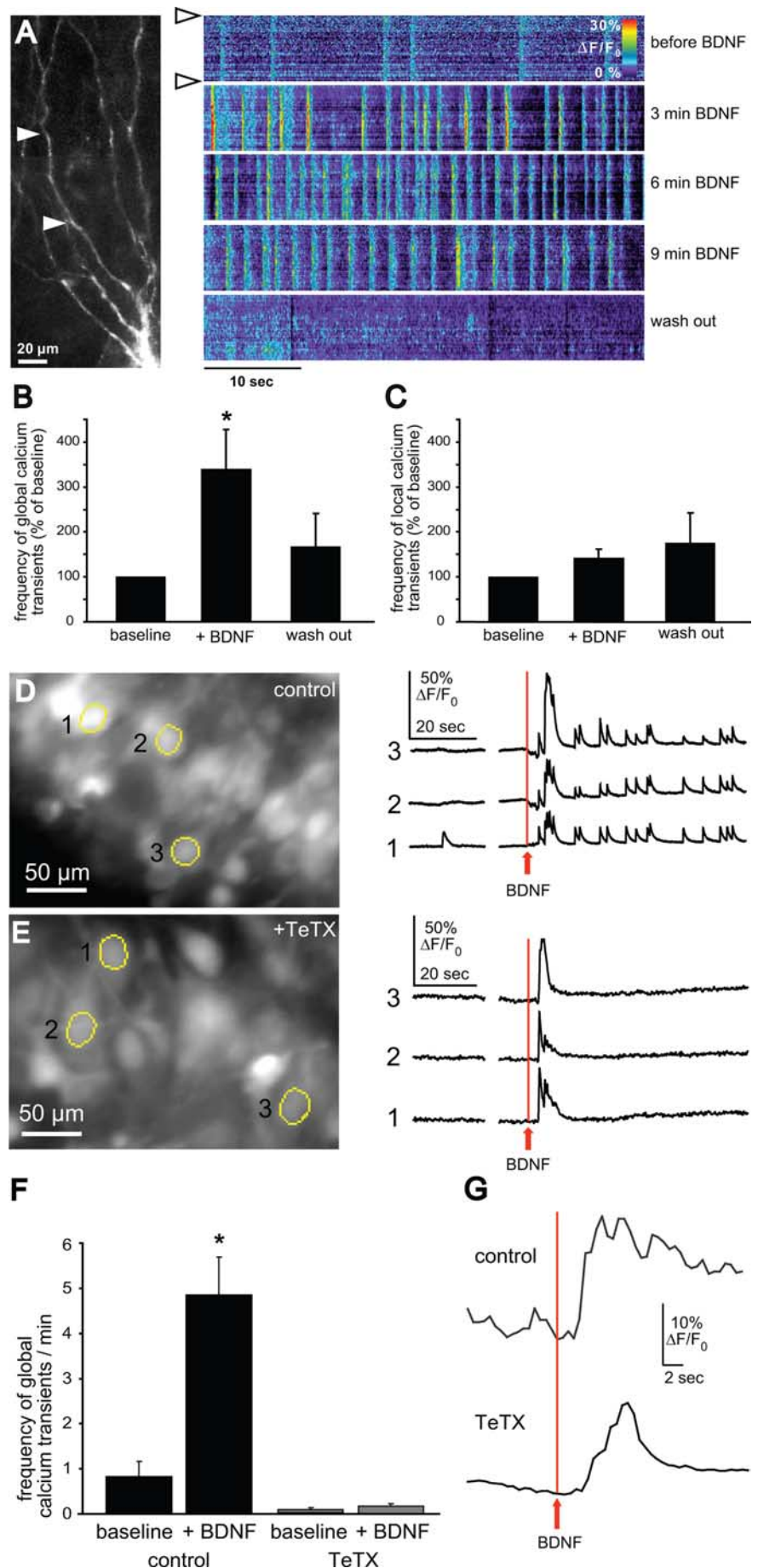
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Figure 2. BDNF bath application causes a strong increase in the frequency of global calcium transients. **A**, Spontaneous $[Ca^{2+}]_i$ changes in the dendritic segment marked with the arrowheads are shown in pseudo line scans as $\Delta F/F_0$. Line scans are shown before and during BDNF bath application (at 3 different time points) and after washout. **B**, The frequency of spontaneously occurring global calcium transients is significantly (*$p < 0.05$) increased during bath application of BDNF ($n = 8$ neurons, values normalized to baseline frequency). **C**, Frequency of spontaneous local calcium transients is not significantly increased during BDNF bath application ($n = 12$ neurons). **D**, Bolus loading of CA3 neurons in control slices shows an acute increase in $[Ca^{2+}]_i$ during BDNF bath application and a delayed increase in correlated network activity (traces correspond to the encircled neurons in the left image). **E**, Neurons in slices incubated for 12 h in TeTX also reveal an immediate increase in $[Ca^{2+}]_i$ to BDNF bath application but otherwise remain silenced. **F**, Frequency of global calcium transients in control- and TeTX-incubated slices before and after BDNF bath application (80 neurons from 8 slices; control slices: before, 0.8 ± 0.3; after, 4.9 ± 0.8 global calcium transients per minute; TeTX-incubated slices: before, 0.07 ± 0.02; after, 0.17 ± 0.06 global calcium transients minute; *$p < 0.05$). **G**, Averaged acute calcium responses to fast BDNF bath application in control slices and in TeTX-incubated slices (80 neurons from 8 slices).
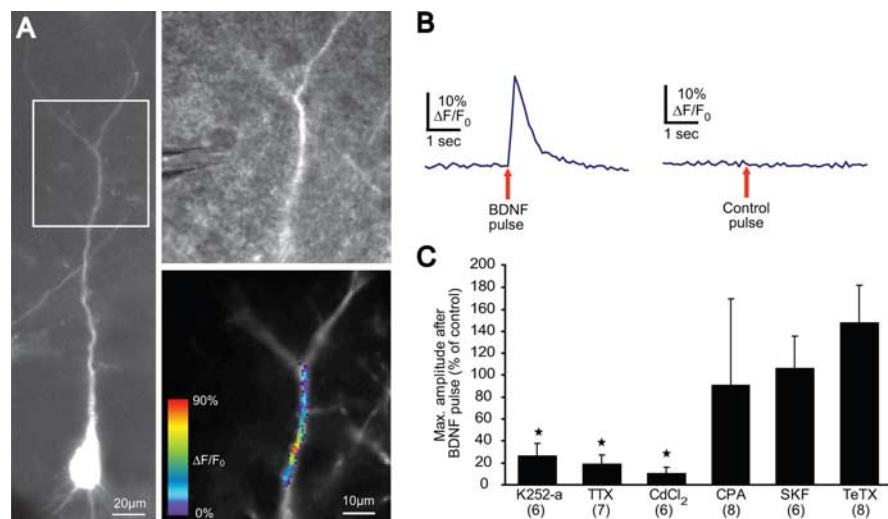
Figure 3. Dendritic calcium transients can be elicited by focal BDNF application and are dependent on activation of TrkB receptors as well as voltage-gated sodium and calcium channels. **A**, An electroporated CA3 neuron with the BDNF application pipette, placed ~20 μm from the dendrite (top right), responds to a single 40 ms BDNF pulse with a strong local increase in $[Ca^{2+}]_i$. **B**, Left trace shows an average of calcium responses to six BDNF pulses applied at the indicated dendritic region in **A**. Right trace shows averaged responses to six pulses of a control solution (heat-inactivated BDNF) in another neuron. **C**, Pharmacology of BDNF-evoked calcium transients: amplitudes of BDNF-evoked calcium transients are shown as percentage of baseline. Bath application of K252-a, TTX, and $CdCl_2$ reduced the amplitude of the calcium transients evoked by local BDNF application significantly (*$p < 0.05$). In contrast, no reduction in the amplitude of the BDNF-induced calcium transients was observed during bath application of CPA, SKF 96365 hydrochloride, or in TeTX incubated slices.

elicited only the immediate calcium response (Fig. 2E,G), demonstrating that the increase in frequency that we observed in control slices after BDNF application was a consequence of a BDNF-induced modulation of network activity. Fast application of control solution (PBS) caused neither an acute rise in the calcium concentration nor an increase in the frequency of calcium transients (data not shown). Together, these observations show that exogenous BDNF has two effects on CA3 neurons: it (1) directly triggers a transient rise in $[Ca^{2+}]_i$ and (2) causes an increase in network activity, probably by modulating synaptic function (Shen et al., 2006; Tyler et al., 2006; Walz et al., 2006).

Focal BDNF application triggers fast and local calcium transients

To further characterize calcium transients evoked directly by BDNF, we tested its specific action on dendrites of neurons by focally applying BDNF via a micropipette using a Picospritzer. We placed a pipette filled with BDNF 10–20 μm away from the dendrite (Fig. 3A) and applied single BDNF pulses (40 ms, 200 ng/ml) to the dendrite of neurons exhibiting spontaneous calcium activity. In ~60% of those neurons, single BDNF pulses elicited dendritic calcium rises (Fig. 3A). We recorded twice for 1 min at 10 Hz with an interval of 3 min between acquisitions. During these two recordings, we applied a total of six BDNF pulses to the dendrite. The calcium responses to the six BDNF pulses were averaged for each cell as shown in the left trace in Figure 3B. We could repetitively evoke fast (rise time <100 ms) and spatially restricted calcium transients. Only cells that responded to at least two of the six BDNF pulses with a calcium rise and that showed an average response >5% $\Delta F/F_0$ were analyzed (n = 48). We performed control experiments to verify that the observed calcium response was specific for the BDNF application: neither PBS plus 0.1% BSA alone (n = 5) nor heat-inactivated BDNF (n = 10) elicited calcium transients in the dendrite (Fig. 3B, right trace). Moreover, focal PBS plus 0.1%

BSA application did not evoke calcium transients in those dendrites that responded to BDNF pulses before and after PBS application (n = 3; data not shown).

Next, we determined the mechanisms of fast BDNF–TrkB-mediated calcium signaling by pharmacologically characterizing exogenously BDNF-evoked calcium transients. We measured the maximal amplitude of the calcium response within 1 s after the BDNF pulse before, during, and after bath applying drugs. K252-a (200 nM) as well as TTX (1 μM) and $CdCl_2$ (5 μM), which block TrkB receptors, voltage-gated sodium and calcium channels, respectively, reduced the maximal amplitude of the calcium response after the BDNF pulse significantly (Fig. 3C). In contrast, neither CPA (20 μM), an agent that interferes with calcium release from internal stores, nor SKF 96365 hydrochloride (3 μM), an unspecific inhibitor of store-operated calcium entry and transient receptor potential channels (Merritt et al., 1990; Li et al., 1999), nor TeTX (20 nM), which blocks presynaptic transmitter release, reduced the amplitude of the calcium response after the BDNF pulse. Together, these pharmacological experiments indicate that activation of the TrkB receptor and voltage-gated sodium as well as calcium channels are required for BDNF-evoked calcium transients.

BDNF-mediated local calcium transients occur at synaptic sites
The observation that spontaneous BDNF-dependent calcium transients were highly localized along the dendrite (Fig. 1) led us to ask whether these transients occur at specific sites, possibly synapses. Using viral infection (Semliki forest virus) (Ehrengruber et al., 1999), we expressed the postsynaptic marker PSD-95:CFP (Graf et al., 2004) in hippocampal neurons. PSD-95:CFP labeling was apparent in the soma and as puncta along the dendrites. We identified presynaptic counterparts of the dendritic PSD-95:CFP puncta with immunohistochemical staining for synapsin. High-magnification confocal reconstruction in three dimensions allowed us to visualize synaptic structures by colocalization analysis. Seventy-four percent of 122 putative PDS-95:CFP puncta were opposed by at least one positive synapsin punctum, whereas 26% did not show a clear colocalization (Fig. 4A). The specificity of colocalization was tested using the reverse order of the synapsin stack rotated by 180°. Here, significantly fewer of the PSD-95 clusters colocalized with presynaptic synapsin puncta (16% colocalization, 84% non-colocalization; n = 8 neurons; $p < 0.05$). This indicates that the majority of the PSD-95:CFP puncta are indeed postsynaptic sites.

After electroporation of the calcium indicator into PSD-95:CFP-expressing cells, we were able to visualize postsynaptic sites along the dendrite and spontaneous calcium transients simultaneously. Cellular morphology and the frequency of spontaneously occurring global and local calcium transients in these transfected neurons were similar to uninfected neurons. We frequently observed spontaneous local calcium transients at PSD-95:CFP-expressing sites along the dendrite (Fig. 4B). We

identified the positions of putative synapses and local calcium transients along the dendrites and calculated the distance between the center of the nearest putative synaptic site and the center of each calcium transient. Almost half of the local calcium rises occurred within a distance of 2 μm of a putative synapse (41 of 99 from 8 neurons) (Fig. 4C), which is consistent with previous observations (Lohmann et al., 2005). The mean distance between visualized PSD-95:CFP sites was 17.3 ± 1.9 μm, which is in good agreement with numbers that can be deduced from electron microscopy studies of CA1 tissue in the hippocampus (Steward and Falk, 1991). To test whether BDNF triggers local calcium transients specifically at PSD-95 sites, we again used function-blocking antibodies against BDNF to interfere with the BDNF–TrkB signaling pathway. We observed that, during BDNF antibody bath application, the frequency of local calcium transients that occurred within a distance of <2 μm from a putative synaptic site was significantly and reversibly reduced to 65.9 ± 16.8% (n = 51) (Fig. 4D, black bars) compared with baseline. At those synapses that exhibited calcium activity during baseline recordings (21 of 51), calcium transients were completely blocked in most cases (17) or were reduced (two) or remained stable (two) during αBDNF application. We calculated that intrinsic BDNF signaling occurred at excitatory synapses at a mean rate of 0.22 transients per minute and synapse. In contrast to the reduced frequency of calcium transients at synapses, we did not find a change in the frequency of local transients that occurred >2 μm away from the nearest synapse during BDNF antibody application (Fig. 4D, gray bars). Furthermore, there was no significant change in the frequency of global calcium rises during BDNF antibody application (1.44 ± 0.51 global transients per minute) compared with baseline conditions (1.94 ± 0.82 global transients per minute; data not shown). Together with the experiments described above, this finding indicates that endogenous BDNF directly triggers local calcium transients but not global calcium rises and that these BDNF–TrkB-dependent local calcium transients occur specifically at synaptic sites.

Local calcium transients elicited at synaptic sites by focal BDNF delivery
Focal BDNF application demonstrated a dendritic mechanism of the BDNF-evoked calcium responses (Fig. 3C). Additionally, we showed that spontaneous local calcium transients occur at PSD-95:CFP sites along the dendrite. Therefore, we tested whether dendrites respond to exogenous BDNF only at postsynaptic sites.
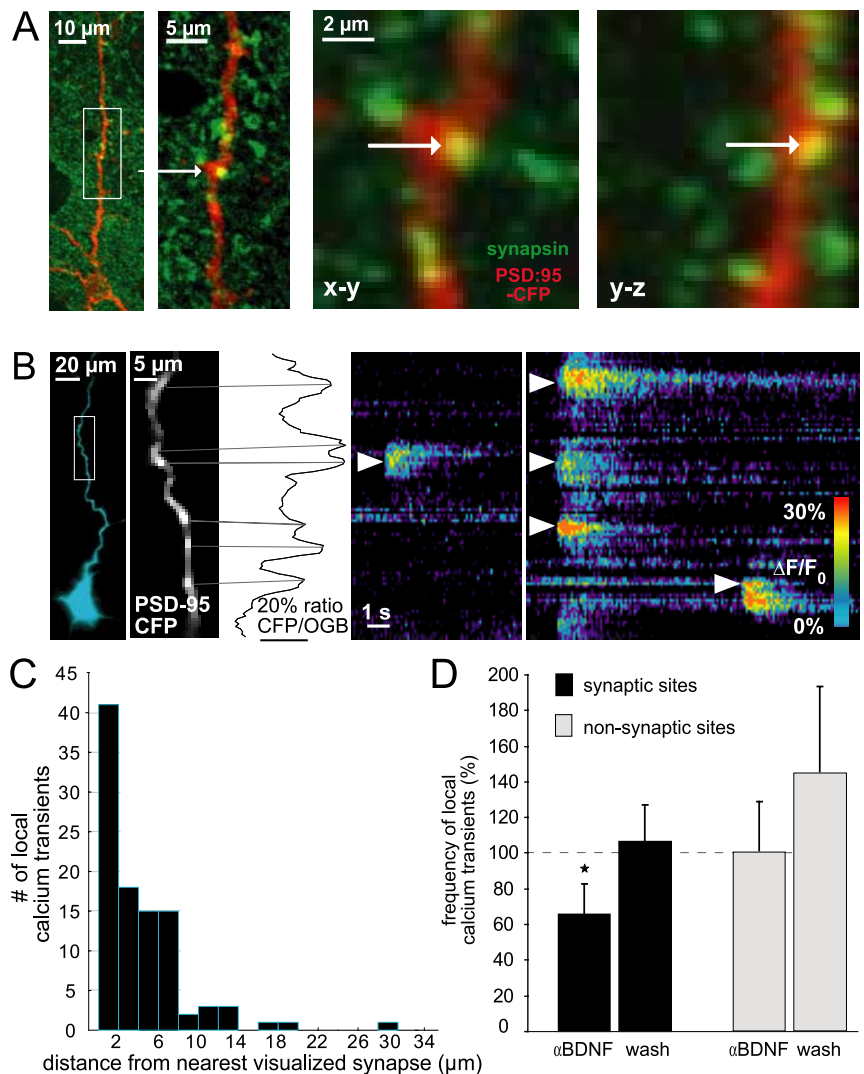

Figure 4. BDNF-mediated local calcium transients occur preferentially at synaptic sites. **A**, Neuron expressing PSD-95:CFP (shown in red) by virus-mediated gene transfer after immunolabeling with anti-synapsin antibodies and high-resolution confocal reconstruction (left, maximum intensity projection of the neuron in red, single plane of synapsin staining in green). Arrow indicates a synapse as PSD-95:CFP and synapsin overlap. High-magnification (x–y) and orthogonal (y–z) views of the marked synapse (yellow pixels in both views). **B**, PSD-95:CFP (blue) expressing hippocampal neuron electroporated with the calcium indicator Oregon Green BAPTA-1 (OGB) (green). Enlarged part of the dendrite shows PSD-95:CFP puncta; the puncta are also revealed in a ratio plot of CFP/Oregon Green BAPTA-1 pixel intensity along the dendrite. Line scan of the enlarged part of the dendrite shows spontaneously occurring local calcium transients at four of the PSD-95:CFP sites (right). **C**, Distribution of spontaneous local calcium transients with respect to putative synaptic sites (99 local calcium signals from 8 neurons). Most transients occur at or very close to the nearest synapse (the mean intersynapse distance of 17.3 ± 1.9 μm agrees with numbers observed in CA1 neurons in the first postnatal week) (Steward and Falk, 1991). **D**, Frequency of spontaneous local calcium transients occurring at a synapse (<2 μm apart) and at nonsynaptic sites (>2 μm apart from the nearest synapse) before, during, and after bath application of BDNF antibodies. The frequency of local calcium transients at synaptic sites, but not at nonsynaptic sites, is significantly reduced during bath application of BDNF antibodies (n = 8 neurons; percentage of baseline; *p < 0.05).

Neurons with spontaneous calcium activity were used for these experiments. The tip of a BDNF-filled glass pipette was placed 10–20 μm away from the PSD-95 site of the dendrite (Fig. 5A, middle). A single BDNF pulse evoked a strong and localized calcium transient exactly at the PSD-95:CFP sites (Fig. 5A, right). The respective line scan indicates that the BDNF-mediated calcium rise started precisely at the two PSD-95 puncta on the dendrite and reaches the maximum change in fluorescence there (Fig. 5A, bottom). Another example of a BDNF pulse, followed immediately by a localized calcium rise at a synaptic site, is shown in Figure 5B. Here, BDNF evoked a larger signal at the PSD-95:
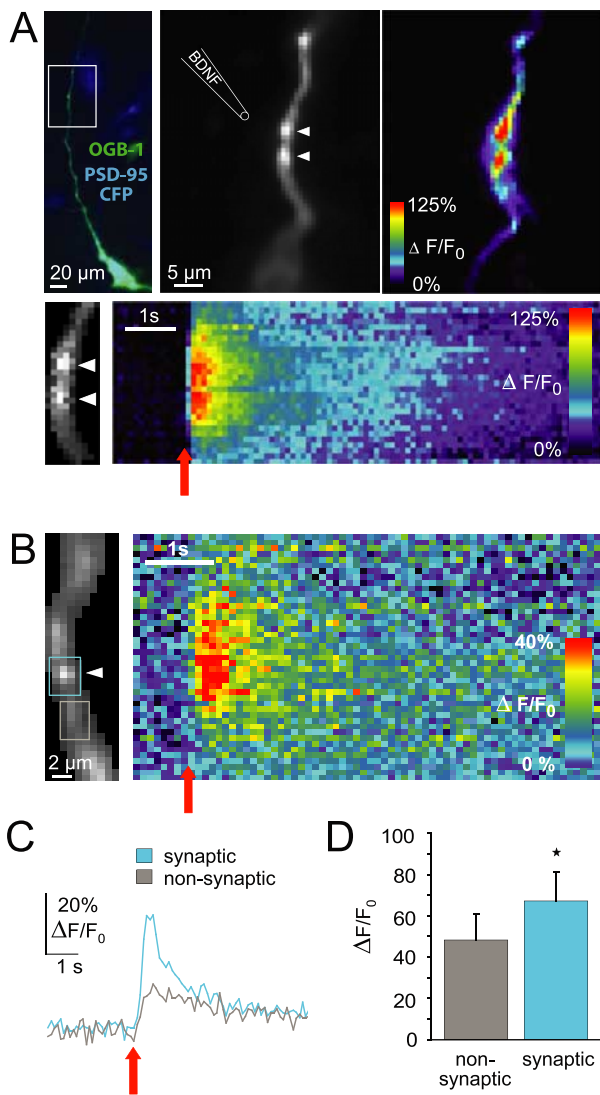
Figure 5. BDNF-evoked local calcium transients can be induced at synaptic sites. **A**, PSD-95:CFP-expressing pyramidal neuron, electroporated with Oregon Green BAPTA-1 (OGB-1) (left); arrowheads mark PSD-95:CFP puncta along the apical dendrite. A BDNF pulse caused a strong calcium rise (represented as $\Delta F/F_0$ values) exactly at the sites of two putative synapses (right). Bottom, Line scan reveals that calcium transient induced by the BDNF pulse (red arrow) starts at the two PSD-95:CFP sites and shows exactly there the highest fluorescence increase. **B**, Another example of a BDNF-evoked calcium transient at a PSD-95:CFP site. **C**, BDNF-evoked calcium transients at a PSD-95:CFP punctum (blue trace) and at an adjacent region (gray trace) indicated in **B**. **D**, Maximal amplitudes of $\Delta F/F_0$ within 1 s after the BDNF pulse at nonsynaptic dendritic sites and at PSD-95:CFP puncta (*$p < 0.05$; $n = 9$).

CFP punctum (blue region) than at the adjacent nonsynaptic dendrite (Fig. 5C, gray region). On average, the amplitude of the $\Delta F/F_0$ signal after the BDNF pulse was significantly higher at the PSD-95:CFP puncta than at the adjacent nonsynaptic dendritic sites (Fig. 5D). These findings show that focal, pulse-like delivery of BDNF evokes localized calcium rises restricted to synaptic sites.

Discussion

We used calcium imaging to investigate directly intrinsic BDNF signaling in developing hippocampal neurons during synapse formation. Our results provide strong evidence that intrinsic BDNF signaling is highly specific in space and time; endogenous BDNF induces fast calcium transients at synapses in developing dendrites. Furthermore, by quantifying the characteristics of BDNF-triggered calcium transients, we determined constraints for the regulation of developmental processes by fast BDNF signaling. The temporal (<1 s) and spatial (<10 μm) characteristics of intrinsic BDNF signaling as well as its relative abundance (>10/h/synapse) render BDNF an ideal signaling molecule for establishing specific synaptic connectivity and functional neuronal networks.

Intrinsic BDNF signaling is fast

Blocking intrinsic BDNF signaling in developing hippocampal slices reduced the frequency of spontaneous fast and locally restricted calcium transients. Conversely, focal application of exogenous BDNF elicited fast local calcium transients. Together, these results show that BDNF is an important mediator of fast local calcium transients in developing dendrites.

What makes BDNF signaling so fast? We investigated the mechanism of BDNF-triggered dendritic calcium transients in developing dendrites of hippocampal neurons and found that they depend on the activation of TrkB and fast opening ion channels, such as voltage-gated sodium and calcium channels. They are not dependent on release of calcium from internal stores. The tetanus toxin experiments exclude the possibility that BDNF may stimulate presynaptic transmitter release, which could in turn induce dendritic calcium transients. Together, our results show that BDNF directly triggers dendritic calcium transients via TrkB and voltage-gated currents. Such a mechanism is reminiscent of the effects of exogenously applied BDNF, which have been described previously. BDNF can cause depolarizations in cultured neurons within a few milliseconds (Kafitz et al., 1999) that require the activation of voltage-dependent sodium channels (Blum et al., 2002) as well as fast calcium transients through the opening of voltage-gated calcium channels (Berninger et al., 1993; Kovalchuk et al., 2002). Our results extend these findings by demonstrating that developing hippocampal neurons intrinsically generate fast BDNF signaling. The onset kinetics of BDNF-evoked calcium transients are faster than 100 ms, the temporal resolution of our recordings, suggesting that they are in the range of several tens of milliseconds or less, which is within the temporal requirements for spike-timing-dependent plasticity in the mature nervous system (Dan and Poo, 2004). There is evidence, however, for a reduced requirement for temporal precision in activity-dependent plasticity during development (0.1–1 s) (Lee et al., 2002; Kasyanov et al., 2004). Therefore, the temporal characteristics of BDNF induced calcium transients (rise time <100 ms, duration ~700 ms) accurately reflect the temporal requirements for activity-dependent synaptic plasticity during development. Furthermore, we show that this phenomenon occurs under natural conditions frequently enough to play a pivotal role in synapse development.

In principle, two scenarios could explain fast intrinsic BDNF signaling: (1) rapid release of BDNF at specific sites, most likely at synapses (see below), triggers a direct response in the dendrite; (2) tonic BDNF release establishes a diffuse distribution of BDNF throughout the tissue and stochastic activation of TrkB receptors generates fast dendritic calcium transients. Our experiments support the first scenario, because fast and localized application of BDNF triggers immediate calcium rises (Figs. 2G, 3, 5). In contrast, our data are not consistent with the second scenario, because we find that the persistent and diffuse presence of BDNF is not an appropriate stimulus for the direct activation of fast calcium rises (Fig. 2C,E). Desensitization of TrkB receptors might prevent a sustained response during long-term BDNF exposure.

From these findings, we infer that the induction of fast calcium transients requires the rapid release of BDNF in close vicinity to the dendrite. In addition, these results support the idea that both endogenous BDNF release and dendritic response are very precisely regulated in time.

Intrinsic BDNF signaling is localized to synapses

We observed that BDNF-evoked calcium transients are localized to specific dendritic sites. Interestingly, simultaneous imaging of putative postsynaptic structures, labeled with PSD-95:CFP, and local calcium transients revealed that BDNF-triggered transients occur primarily or exclusively at synapses.

One potential caveat of virally overexpressing synaptic PSD-95 is that it may affect health of infected neurons. Additionally, it might change signaling as it increases the number of synaptic AMPA receptors as shown previously (Schnell et al., 2002). The increase in synaptic AMPA receptors will most likely not influence the localization of TrkB because no direct interaction between TrkB and AMPA receptors or PSD-95 has been shown. Furthermore, we observed no difference in the structure or function between transfected and wild-type cells, indicating that overexpression did not affect viability or signaling. (1) The cellular morphology of transfected pyramidal neurons was not different from nontransfected neurons. (2) The frequencies of spontaneously occurring global and local calcium transients were similar in normal and overexpressing neurons. (3) The proportion of local calcium transients occurring at synapses was similar to that found in a previous study using postfixation immunohistochemistry instead of PSD-95:CFP for labeling synapses (Lohmann et al., 2005). (4) The proportion of local calcium transients that were blocked by interfering with BDNF signaling was similar in normal and transfected cells. Together with our finding that most PSD-95:CFP puncta were opposed by synapsin-positive structures, these observations confirm that virally overexpressed PSD-95:CFP is an appropriate label for identifying postsynaptic signaling.

We found BDNF-induced calcium transients at synaptic sites during intrinsic BDNF signaling and after focal delivery of BDNF. The second observation implicates that dendrites are particularly sensitive to BDNF at synapses. The most straightforward explanation would be the specific presence of TrkB at the synapse. This fits well with an immunoelectron microscopical study that reported TrkB immunoreactivity predominantly at synapses within the dendrites of hippocampal pyramidal neurons (Drake et al., 1999). TrkB, which resides at nonsynaptic sites along dendrites, is mostly confined to intracellular membranes (Drake et al., 1999), suggesting that functional receptors are enriched at synaptic sites (Gomes et al., 2006). This predominantly synaptic distribution of TrkB likely underlies the local specificity of BDNF-evoked calcium transients observed here.

Although our results implicate that endogenous BDNF is released near the synapse and directly triggers calcium transients at the postsynapse, it is not clear whether BDNF originates from the presynapse or postsynapse. Evidence for both modes of release has been obtained (Hartmann et al., 2001; Kohara et al., 2001; Lessmann et al., 2003; Gartner et al., 2006). Independently of the site of release, it has been shown previously that BDNF regulates neuronal development on a local as well as on a global level. The here identified intrinsic form of fast BDNF signaling occurs at synaptic sites and extends only a few micrometers along the dendrite, much less than the average distance between excitatory synapses at these early stages of synapse development. This observation implicates intrinsic BDNF signaling in regulating the development of single synapses, possibly by mediating synaptic maturation (Fig. 3) (Rutherford et al., 1998; Itami et al., 2003; Walz et al., 2006). In addition, because TrkB activates diverse intracellular signaling pathways, the intriguing possibility arises that BDNF–TrkB signaling activates parallel pathways to regulate local and global as well as rapid and slower aspects of neuronal development differentially.

Our results also indicate that BDNF is one of several factors, which contribute to local dendritic calcium activity, because only a fraction of local dendritic calcium transients is blocked by interfering with intrinsic BDNF signaling. We conclude that developing neurons are able to respond to a number of different extracellular cues during development and that they integrate those by generating very specific patterns of local and global calcium transients. Such multifactorial calcium transients may regulate structural development and synapse formation and eventually help establish the complex neuronal networks of the brain.

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