

Activity-Dependent Release of Endogenous BDNF From Mossy Fibers Evokes a TRPC3 Current and Ca²⁺ Elevations in CA3 Pyramidal Neurons

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Li Y, Calfa G, Inoue T, Amaral MD, Pozzo-Miller L. Activity-dependent release of endogenous BDNF from mossy fibers evokes a TRPC3 current and Ca²⁺ elevations in CA3 pyramidal neurons. *J Neurophysiol* 103: 2846–2856, 2010. First published March 10, 2010; doi:10.1152/jn.01140.2009. Multiple studies have demonstrated that brain-derived neurotrophic factor (BDNF) is a potent modulator of neuronal structure and function in the hippocampus. However, the majority of studies to date have relied on the application of recombinant BDNF. We herein report that endogenous BDNF, released via theta burst stimulation of mossy fibers (MF), elicits a slowly developing cationic current and intracellular Ca²⁺ elevations in CA3 pyramidal neurons with the same pharmacological profile of the transient receptor potential canonical 3 (TRPC3)-mediated *I*_{BDNF} activated in CA1 neurons by brief localized applications of recombinant BDNF. Indeed, sensitivity to both the extracellular BDNF scavenger tropomyosin-related kinase B (TrkB)-IgG and small hairpin interference RNA-mediated TRPC3 channel knockdown confirms the identity of this conductance as such, henceforth-denoted MF-*I*_{BDNF}. Consistent with such activity-dependent release of BDNF, these MF-*I*_{BDNF} responses were insensitive to manipulations of extracellular Zn²⁺ concentration. Brief theta burst stimulation of MFs induced a long-lasting depression in the amplitude of excitatory postsynaptic currents (EPSCs) mediated by both AMPA and *N*-methyl-D-aspartate (NMDA) receptors without changes in the NMDA receptor/AMPA receptor ratio, suggesting a reduction in neurotransmitter release. This depression of NMDAR-mediated EPSCs required activity-dependent release of endogenous BDNF from MFs and activation of Trk receptors, as it was sensitive to the extracellular BDNF scavenger TrkB-IgG and the tyrosine kinase inhibitor k-252b. These results uncovered the most immediate response to endogenously released—native—BDNF in hippocampal neurons and lend further credence to the relevance of BDNF signaling for synaptic function in the hippocampus.

INTRODUCTION

A critical function of the CNS is the ability to assimilate and respond to a range of environmental stimuli; these tasks are reliably accomplished by modulation of synaptic structure and function in response to various neuronal firing patterns. Brain-derived neurotrophic factor (BDNF) is an important modulator of activity-dependent synaptic plasticity in the CNS (Bramham and Messaoudi 2005; Poo 2001), particularly in the hippocampus, wherein resides the foundation for learning and memory. Indeed, it is this region that exhibits some of the highest levels

of BDNF protein expression in the brain. Once thought to be important only in developmental processes, BDNF is now a well-known modulator of the synaptic landscape in the postnatal brain, participating in the induction of long-term plasticity of excitatory synaptic transmission in the hippocampus (Figurov et al. 1996; Huber et al. 1998; Kang and Schuman 1995; Korte et al. 1995; Kumura et al. 2000; Minichiello et al. 1999; Patterson et al. 1996) as well as in the density and morphology of dendritic spines, an effect that ultimately translates into a change in the number of excitatory synapses (Tolwani et al. 2002; Tyler and Pozzo-Miller 2001). Moreover, the induction of long-term potentiation (LTP) in area CA1 of the hippocampus is impaired in mice lacking *Bdnf* (Korte et al. 1995; Patterson et al. 1996; Pozzo-Miller et al. 1999) or its receptor *Trkb* (Minichiello et al. 1999).

Although numerous BDNF actions have been identified, the underlying mechanisms that bring about these changes are not clearly defined. It is well established that BDNF activation of tropomyosin-related kinase B (TrkB) receptors sets in motion three major intracellular signaling cascades: the Ras-Raf MAPK/ERK cascade, the PI3 kinase cascade, and the PLCγ-IP₃ cascade (Segal and Greenberg 1996). The Ras-Raf MAPK/ERK cascade is largely responsible for changes in gene transcription and is necessary for the induction of LTP, hippocampal-dependent associative learning, and BDNF-induced spine formation (Alonso et al. 2004; Atkins et al. 1998; Bading et al. 1993; English and Sweatt 1997; Rosen et al. 1994). At presynaptic terminals in area CA1 of the hippocampus, MAPK/ERK-dependent phosphorylation of synapsin-I results in a higher density of docked neurotransmitter vesicles in glutamatergic terminals, increased frequency of miniature excitatory postsynaptic currents (mEPSC), increased paired-pulse facilitation, facilitated neurotransmitter release during high-frequency activity with consequent increases in LTP, and enhancements in hippocampus-dependent learning (Kushner et al. 2005). The PI3 kinase cascade is responsible for dendritic targeting of BDNF and TrkB mRNA (Righi et al. 2000) and has been implicated in hippocampal-dependent spatial learning (Mizuno et al. 2003). Last, the PLCγ-IP₃ cascade also plays a critical role in hippocampal synaptic plasticity as demonstrated by the lack of CA1 LTP in conditional knockout mice carrying a deletion of the PLC-γ adaptor site in Trk receptors (Minichiello et al. 2002).

But what is the most immediate effect of BDNF of these several signaling possibilities? Kafitz et al. (1999) showed that brief (millisecond) recombinant BDNF pulses to neurons in acute brain slices activated a TTX-insensitive, STX-sensitive

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fast Na^+ current later proposed to be mediated by $\text{Na}_v1.9$ channels (Blum et al. 2002). On the other hand, Li et al. described the activation of a much slower nonselective cationic conductance in acutely dissociated pontine neurons by bath applied recombinant BDNF— I_{BDNF} —which required TrkB receptors, $\text{PLC}\gamma\text{-IP}_3$, intracellular Ca^{2+} elevations, and canonical transient receptor potential (TRPC) channel activity (Li et al. 1999). TRPC ion channels are activated by stimulation of Gq/G11-type G protein-coupled receptors and by receptor tyrosine kinases such as tropomyosin related kinase (Trk) receptors, leading to PLC-mediated formation of diacylglycerol (DAG) and IP_3 , followed by activation of IP_3 receptors in intracellular Ca^{2+} stores (Clapham 2003; Montell et al. 2002). We recently described that recombinant BDNF also activates I_{BDNF} in CA1 pyramidal neurons of hippocampal slices and that it requires channels containing TRPC3 subunits (Amaral and Pozzo-Miller 2007a). Both the $\text{PLC}\gamma\text{-IP}_3$ and the PI3 kinase cascades play important roles in the activation and persistence of I_{BDNF} , mobilizing Ca^{2+} from intracellular stores and facilitating transport of TRPC3 containing vesicles to the plasma membrane, respectively (Amaral and Pozzo-Miller 2007a). Moreover, TRPC3 channels are required for BDNF-induced changes in dendritic spine density at CA1 neurons. The aforementioned observations were characterized, however, following application of recombinant BDNF. To confirm that natively expressed endogenous BDNF activates such a membrane conductance, we used theta burst stimulation (TBS), which is known to be an effective stimulus pattern for releasing endogenous BDNF from cultured hippocampal cells (Balkowiec and Katz 2002). Indeed, TBS of Schaffer collaterals in the absence of glutamate and GABA receptor activity evoked a membrane conductance in CA1 neurons that was sensitive to the BDNF scavenger TrkB-IgG, the membrane tyrosine kinase inhibitor k-252a, and the TRPC/SOC (store-operated channels) channel inhibitor SKF-96365 (Amaral and Pozzo-Miller 2007a). Taken together with the dendritic Ca^{2+} signals associated with I_{BDNF} (Amaral and Pozzo-Miller 2007b), which potentially enable cytoskeletal rearrangements and dendritic spine morphogenesis, we concluded that I_{BDNF} is one of the earliest signaling steps in the BDNF/TrkB cascade (Amaral et al. 2007).

We now focus on the mossy fiber (MF) pathway in area CA3 because these fibers contain the highest levels of BDNF in the brain (Conner et al. 1997; Danzer and McNamara 2004). As we have shown previously in area CA1 (Amaral and Pozzo-Miller 2007a,b), BDNF released from MFs during TBS evokes a membrane current in CA3 neurons that is mediated by TRPC3 channels and associated with Ca^{2+} influx, which we termed MF- I_{BDNF} . Consistent with such activity-dependent release of BDNF, these MF- I_{BDNF} responses were insensitive to manipulations of extracellular Zn^{2+} concentration. In addition a single brief TBS of MFs induced a long-lasting depression of the amplitude of EPSCs mediated by AMPA receptors (AMPA) and NMDA receptors (NMDAR) evoked in CA3 neurons, which is associated with changes in the coefficient of variance (CV^2) but not in the NMDAR/AMPA ratio. TBS-induced depression of NMDAR-mediated EPSCs at MF-CA3 synapses was sensitive to TrkB-IgG and k-252b, indicating a requirement of extracellular endogenous BDNF and TrkB receptor activation. This study yields novel information regarding the role of endogenous BDNF function in the hippocampus, especially its availability for activity-dependent release and subsequent activation of TRPC mem-

brane currents and intracellular Ca^{2+} elevations, as well as its role in the modulation of excitatory synaptic strength at MF-CA3 synapses.

METHODS

Organotypic slice cultures

Hippocampi were dissected from postnatal day 7–11 Sprague Dawley rats (Harlan, Indianapolis, IN, or Charles River, Wilmington, MA) and cut transversely into $\sim 500\text{-}\mu\text{m}$ -thick slices using a custom-made wire-slicer fitted with $20\text{-}\mu\text{m}$ -thick Pt wire (Pozzo-Miller et al. 1995). Hippocampal slices were individually plated on Millicell-CM filter inserts (Millipore, Billerica, MA) and cultured in 36°C -5% CO_2 , 98% relative humidity incubators (Thermo-Forma, Waltham, MA). Slices were maintained in culture media (Neurobasal-A plus B27, Invitrogen, Carlsbad, CA) containing 20% equine serum for the first 4 days in vitro (div). To avoid the confounding effects of hormones and growth factors in the serum, its concentration was gradually reduced over a period of 48 h starting at 4 div (24 h each in 10 and 5% serum). After a period of 24 h in serum free media (Neurobasal-A plus B27), 7–10 div slices were used for electrophysiology (Tyler and Pozzo-Miller 2001). Some slice cultures remained in serum containing culture media (20% equine serum) as described in the original publications (Gahwiler 1981; Pozzo-Miller et al. 1993; Stoppini et al. 1991; Yamamoto et al. 1989). All procedures followed national and international ethics guidelines and were annually reviewed and approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham.

Acute slices

Hippocampi were dissected from postnatal day 45–60 C57BL/6 wild-type mice, placed in ice-cold modified artificial cerebrospinal fluid (ACSF, see following text), and cut transversely into $400\text{-}\mu\text{m}$ -thick slices using a vibrating blade microtome (VT1200S, Leica, Nussloch, Germany). For the dissection and storage of acute slices, the modified ACSF contained (in mM) 87 NaCl, 75 sucrose, 2.5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 0.5 CaCl_2 , 7 MgCl_2 , and 10 glucose (bubbled with 95% O_2 -5% CO_2 ; pH 7.4). Slices were first transferred to this modified ACSF warmed to $\sim 32^\circ\text{C}$ for 30 min and then kept at room temperature in modified ACSF until use for electrophysiology.

Simultaneous electrophysiology and Ca^{2+} imaging

Individual 7–10 div slice cultures or acute slices were transferred to a recording chamber mounted on a fixed-stage upright microscope (Zeiss Axioskop FS, Oberkochen, Germany) and continuously perfused (2 ml/min) with oxygenated ACSF at room temperature (24°C). For slice cultures, ACSF contained (in mM): 124 NaCl, 2 KCl, 1.24 KH_2PO_4 , 1.3 MgSO_4 , 17.6 NaHCO_3 , 2.5 CaCl_2 , and 10 glucose (adjusted to 310–320 mOsm with sucrose); for acute slices, ACSF contained (in mM) 125 NaCl, 5 KCl, 1.25 NaH_2PO_4 , 25 NaHCO_3 , 2 CaCl_2 , 1 MgCl_2 , and 25 glucose (adjusted to 300–320 mOsm with sucrose); both ACSF solutions were continuously bubbled with 95% O_2 -5% CO_2 (pH 7.4). Superficial pyramidal neurons in CA3 stratum pyramidale were visualized with a water-immersion $\times 63$ objective (0.9 NA) using IR-DIC microscopy (Dodt and Zieglgänsberger 1990). Simultaneous whole cell recording and microfluorometric Ca^{2+} imaging was performed as described (Petrozzino et al. 1995; Pozzo-Miller 2006; Pozzo-Miller et al. 1996). Briefly, unpolished patch pipettes contained (in mM) 120 Cs-gluconate, 17.5 CsCl (or KCl), 10 Na-HEPES, 4 Mg-ATP, 0.4 Na-GTP, 10 Na_2 creatine phosphate, 2 QX314, and 0.2 Na-EGTA (replaced by 0.2 mM fura-2 for Ca^{2+} imaging); 290–300 mOsm; pH 7.2 (resistance: 3–4 M Ω). Membrane currents were recorded in the voltage-clamp mode at a holding potential of -65 mV using an Axopatch-200B amplifier (Molecular

Devices, Sunnyvale, CA), filtered at 2 kHz, and digitized at 10 kHz. Recordings were accepted only if access resistance was ≤ 25 M Ω . Input resistance (R_i) was measured with hyperpolarizing voltage pulses (50 ms, 20 mV), and cells were discarded if any whole cell parameter (i.e., C_m , R_i , R_s) changed by $\geq 20\%$ during the course of an experiment. A subsample of CA3 pyramidal neurons had R_i of 275.3 ± 77.9 (SD) M Ω and whole cell capacitances of 135.9 ± 30.4 (SD) pF under the preceding recording conditions ($n = 31$). To reduce the contribution of voltage-gated Ca^{2+} channels during simultaneous whole cell recording and Ca^{2+} imaging, neurons were voltage clamped at +40 mV (Malinow et al. 1994; Perkel et al. 1993; Pozzo-Miller et al. 1996). Fura-2 (Invitrogen) was alternatively excited at 360 and 380 nm using a monochromator (Polychrome-IV, TILL Photonics, Munich, Germany), and its emission (>510 nm) was filtered and detected with a frame-transfer cooled CCD camera (Quantix-57, Photometrics/Roper, Duluth, GA); digital image pairs were acquired every 4 s (50 ms exposures for $\sim 100 \times 200$ pixel subarrays). Background-subtracted fluorescence intensity measurements were obtained within regions of interest (ROIs) defined over apical primary and secondary dendrites, as well as somata. The average ratio of 360 and 380 nm fluorescence within each ROI was used as an estimate of intracellular Ca^{2+} concentration as these two parameters are directly proportional to each other (Grynkiewicz et al. 1985). Electrical and optical data were simultaneously acquired on a single G4 Macintosh computer (Apple) running custom-written software (TIWorkBench, written by T. Inoue).

Afferent fiber stimulation was performed with an extracellular patch pipette filled with buffered and oxygenated ACSF (~ 2 M Ω) positioned within either the granule cell layer or s. lucidum to stimulate the MFs. Square constant-current pulses of 100 μs duration were produced by an isolated stimulator (ISO-Flex, AMPI, Jerusalem, Israel). High-frequency afferent stimulation was delivered as a theta burst pattern consisting of five bursts at 5 Hz, each burst having four pulses at 100 Hz. A subthreshold concentration of the Na^+ channel blocker TTX (10 nM) was included in the ACSF to reduce membrane excitability and prevent polysynaptic responses, common in organotypic slices. The ACSF contained the GABA_A receptor antagonist picrotoxin (50 μM), while the K^+ channels coupled to GABA_B receptors were blocked by intracellular Cs^+ . The ACSF also contained the noncompetitive antagonists of AMPAR and NMDAR, GYKI-52466 (20 μM) and MK-801 (20 μM), respectively, as well as the antagonist of group-I mGlu receptors LY-367385 (100 μM) and L-type Ca^{2+} blocker nimodipine (20 μM); additional experiments were performed with the competitive antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) and D,L-2-amino-5-phosphonovaleric acid (D,L-APV, 100 μM). Membrane currents observed in the presence of GYKI-52466 were 111.8 ± 12.5 pA ($n = 12$), whereas currents in the presence of CNQX were 109.0 ± 11.9 pA ($n = 21$; $P = 0.8803$). The current intensity for afferent stimulation (between 10 and 50 μA) was never larger than 10 times that required to evoke maximal AMPAR-mediated fast EPSCs at -65 mV.

Human recombinant mature BDNF (100 $\mu\text{g}/\text{ml}$; Promega; Madison, WI) dissolved in 0.0001% bovine serum albumin was applied from glass pipettes (~ 5 M Ω) using a Pressure System IIe (Toohey, Fairfield, NJ), as described (Amaral and Pozzo-Miller 2007a,b). The application pipette was positioned ~ 100 μm above the slice and ~ 200 μm away from the soma of the CA3 neuron under recording to avoid pressure and mechanical artifacts and aimed at its apical dendrites within s. lucidum (~ 150 μm from the soma) against the direction of ACSF perfusion flow. Every pulse of 25–30 s duration at 20–30 psi delivered a maximum of 2 μl of the BDNF solution on top of the slice.

TRPC3 RNA interference

Custom HuSH small hairpin interference RNA (shRNA) constructs (OriGene, Rockville, MD) were designed to target TRPC3 channel subunits (Gene Bank Accession No. J05181). Short hairpin RNAs

encoding the following target sequence 5'-GAGTTCAAGAATGAC-TACAGGAAGCTCTC 3' were introduced by transfection with the HuSH construct. The same vector also contained the coding sequence for GFP, and its fluorescence enabled identification of transfected neurons before whole cell recording as described (Amaral and Pozzo-Miller 2007a).

Particle-mediated gene transfer

Hippocampal slices were transfected with a HuSH expression plasmid (OriGene) encoding green fluorescent protein (GFP) and the TRPC3-specific shRNA sequence. A custom-modified Helios gene gun (Bio-Rad; Hercules, CA) was used to perform the biolistic transfection following established protocols (Alonso et al. 2004; Lo et al. 1994). Briefly, the TRPC3-specific shRNA expression vector was precipitated onto 1.6 μm colloidal gold and this mixture was coated onto Tefzel tubing using 0.06 mg/ml polyvinylpyrrolidone. Slices were bombarded using He pressure at 100 psi from a distance of 15 mm. For negative control experiments, gene transfer was performed as in the preceding text using a random shRNA or only enhanced yellow fluorescent protein, except the plasmid encoding eYFP was precipitated onto 1.6 μm colloidal gold at a ratio of 50 μg of DNA to 25 mg of gold.

Drugs

Some compounds were dissolved in DMSO (0.01%) and others directly into the ACSF or intracellular solution; vehicle controls using 0.01% DMSO were routinely performed. When compounds were included in the intracellular solution (e.g., k-252b), a diffusion period of 5–10 min was allowed to elapse before the start of the experiment. All of the chemicals used for these experiments were obtained from Sigma (St. Louis, MO), Calbiochem (San Diego, CA), or Tocris (Ellisville, MO).

Coefficient of variation analysis

The coefficient of variation (CV²) of evoked AMPAR- and NMDAR-mediated EPSCs was calculated after subtraction of the recording noise variance (σ^2): $[(\sigma)^2 - (\sigma \text{ of noise})^2]/\text{mean}^2$, as described (Bender et al. 2009). The fractional change in CV² ($r = \text{CV}^2_{\text{before TBS}}/\text{CV}^2_{\text{after TBS}}$) was plotted against a synaptic gain factor ($f = \text{EPSC amplitude}_{\text{after TBS}}/\text{EPSC amplitude}_{\text{before TBS}}$), as described (Faber and Korn 1991; Shen and Horn 1996).

Statistical analysis

Averages of multiple measurements are presented as means \pm SE; mean \pm SD was used to calculate the coefficient of variation (CV). Data were statistically analyzed using unpaired Student's *t*-test, ANOVA test, and pair-wise correlation and linear regression using the Prism software package (GraphPad Software, San Diego, CA). Probability values <0.05 were considered statistically significant (i.e., $P < 0.05$, $<5\%$ probability that the observations are due to chance). When different than this cutoff value, the actual *P* values ≤ 4 decimal points are given in RESULTS (rather than just the statement "greater than" or "less than") to provide readers with more detailed information regarding the outcome of the statistical analyses. Compromise power analyses were performed to determine the statistical power given the number of observations, sample means and SDs, using G*Power (Erdfelder et al. 1996). These power analyses yielded values of statistical Power ($1 - \beta$; where β is the type-II error) larger than 0.95 (i.e., 95% confidence of accepting the null hypothesis when is true).

RESULTS

Activity-dependent release of BDNF from MFs evokes MF- I_{BDNF} , a slow membrane current in CA3 pyramidal neurons

We recently showed that stimulation of afferent Schaffer collaterals activated TRPC-like nonselective cationic currents in CA1 pyramidal neurons by means of the release of endogenous—native—BDNF (Amaral and Pozzo-Miller 2007a). We now focus on the MF pathway in CA3 because these fibers contain the highest levels of BDNF in the brain (Conner et al. 1997; Danzer and McNamara 2004). In the following experiments, the identity of MF-mediated responses was routinely confirmed using the group-II mGluR agonist DCG-IV (Toth et al. 2000). Indeed, EPSC amplitudes decreased significantly after bath application of 1 μ M DCG-IV (76.00 ± 5.42 vs. 204.52 ± 5.59 pA, $n = 5$; $P < 0.001$; Fig. 1A). TBS has been shown to be an effective stimulation pattern to release GFP-tagged BDNF (Hartmann et al. 2001) or endogenous BDNF from cultured neurons (Balkowiec and Katz 2002). Consistently, stimulation of MFs in CA3 s. lucidum of 7–10 div organotypic slice cultures with a single TBS train evoked slowly activating inward currents in CA3 pyramidal neurons voltage clamped at -65 mV in the presence of ionotropic and metabotropic glutamate and GABA receptor antagonists (20 μ M GYKI-52466 or 20 μ M CNQX for AMPARs; 20 μ M MK-801 or 50 μ M D,L-APV for NMDARs; 100 μ M LY-367385 for mGluR1/5; 50 μ M picrotoxin for GABA_ARs; Cs⁺ intracellular solution to block GABA_B-coupled K⁺ channels; 10 nM TTX to reduce polysynaptic activity). The average amplitude of MF-induced I_{BDNF} was 109.02 ± 11.89 pA, and they peaked 1.47 ± 0.23 s following the onset of afferent stimulation ($n = 21$; Fig. 1B). MF- I_{BDNF} observed in the presence of GYKI-52466 was 111.8 ± 12.5 pA ($n = 12$), whereas MF- I_{BDNF} in the presence of CNQX was 109.0 ± 11.9 pA ($n = 21$; $P = 0.8803$), demonstrating that kainate receptors did not contribute to the measured responses. The amplitude of MF- I_{BDNF} was very stable, lacking any significant “rundown”

during recording times ≤ 90 min from whole cell access (Fig. 1B). Similar responses were observed in acute slices from 45- to 60-day-old C57BL/6 wild-type mice (117.83 ± 11.85 pA, $n = 9$; $P = 0.6469$ compared with MF- I_{BDNF} from organotypic cultures from rat hippocampal slices; Supplemental Fig. S1A).¹

TrkB-IgG, a chimeric recombinant protein that fuses together the ligand-binding domain of the TrkB receptor with the Fc domain of human IgG (Shelton et al. 1995), was used as an extracellular scavenger to implicate endogenous BDNF binding to membrane receptors in MF- I_{BDNF} activation. Consistent with observations in CA1 (Amaral and Pozzo-Miller 2007a), TrkB-IgG (1 μ g/ml, equivalent to 3.8 nM of the receptor dimer) significantly reduced MF- I_{BDNF} amplitude (20.49 ± 6.4 pA, $n = 10$; $P < 0.001$ vs. controls; Fig. 1B). As controls, a nonspecific human IgG (1 μ g/ml) did not affect MF- I_{BDNF} (147.26 ± 39.25 pA, $n = 7$; $P > 0.05$ vs. controls; Fig. 1B). In addition, increasing stimulus intensities evoked larger currents (Fig. 1C), suggesting an activity-dependent process.

Vesicular Zn²⁺ released during MF stimulation does not contribute to MF- I_{BDNF}

At glutamatergic synapses, neurotransmitter vesicles often-times store and release Zn²⁺ alongside their glutamate content, thereby increasing the extracellular concentration of Zn²⁺ (Frederickson et al. 2005). Nowhere is this more apparent than in CA3 s. lucidum, the brain region with the highest vesicular Zn²⁺ content. In addition to its action on NMDA receptors (Mayer and Vyklicky 1989; Mayer et al. 1989), Zn²⁺ also modulates LTP induction at MF-CA3 synapses: removal of free Zn²⁺ with Ca-EDTA prevented LTP induction, and exogenous addition of Zn²⁺ potentiated MF-CA3 extracellular field EPSPs (Li et al. 2001). Because synaptically released Zn²⁺ was recently proposed to trans-activate TrkB receptors in CA3 (Huang et al. 2008), we experimentally altered its extracellular concentration to determine whether MF- I_{BDNF} is in any way influenced by vesicular Zn²⁺ released during TBS of MFs.

¹ The online version of this article contains supplemental data.

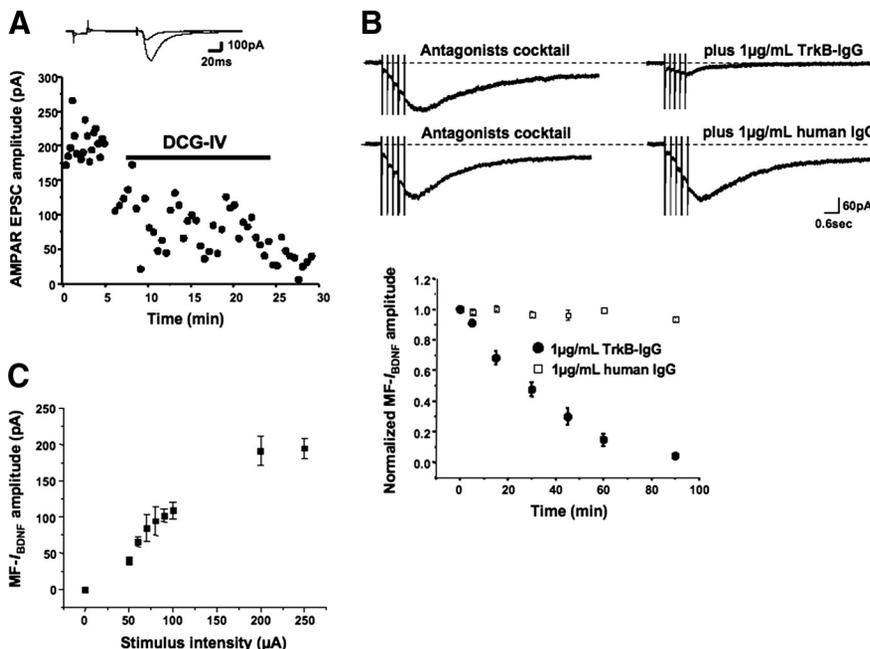


FIG. 1. Release of endogenous brain-derived neurotrophic factor (BDNF) from mossy fibers (MFs) activates MF- I_{BDNF} , a nonselective cationic current in CA3 pyramidal neurons. *A*: representative examples of AMPA receptor (AMPA)-mediated excitatory postsynaptic currents (EPSCs) evoked in CA3 pyramidal neurons by MF stimulation, before and after application of DCG-IV. *B*: representative examples of slow currents evoked by theta burst stimulation (TBS) of MFs in CA3 pyramidal neurons in the presence of antagonists of iono- and metabotropic glutamate receptors as well as GABA_A receptors (left). The MF-evoked current (MF- I_{BDNF}) in the same cell was significantly reduced by the extracellular BDNF scavenger tropomyosin-related kinase B (TrkB)-IgG, but not by human control IgG (right). *C*: input-output curve of slow currents evoked by TBS of MFs.

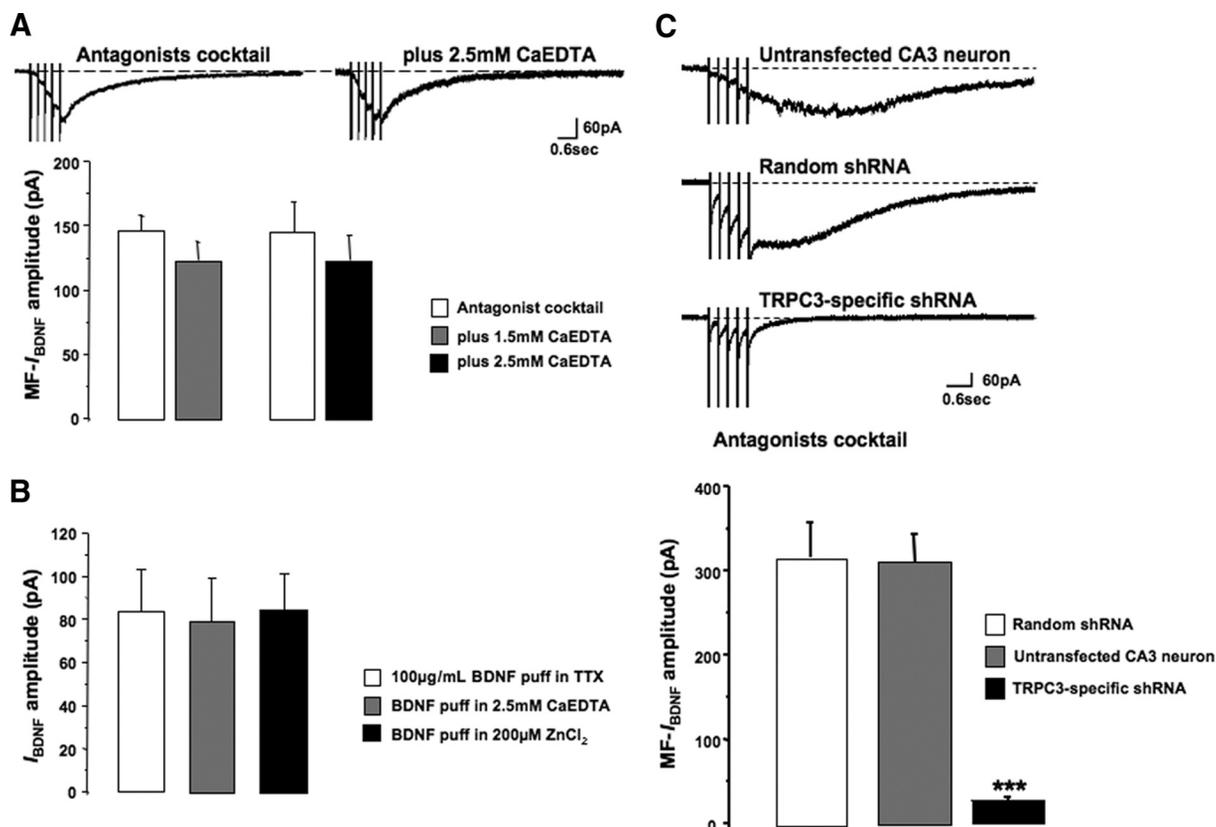


FIG. 2. Vesicular Zn^{2+} released during MF stimulation does not contribute to MF- I_{BDNF} . *A*: Ca-EDTA did not affect MF- I_{BDNF} . *B*: neither Ca-EDTA nor $ZnCl_2$ affected I_{BDNF} , the membrane currents evoked by direct application of recombinant BDNF to apical dendrites (in TTX). *C*: CA3 pyramidal neurons transfected with TRPC3 small hairpin interference RNA (shRNA) show smaller currents than untransfected CA3 neurons in the same slice and cells transfected with a random shRNA (which show currents similar to untransfected cells).

Addition of 1.5 mM of the membrane impermeable Zn^{2+} chelator Ca-EDTA did not affect MF- I_{BDNF} (123.66 ± 13.81 pA, $n = 18$; $P > 0.05$ compared with control responses). Similar results were obtained using 2.5 mM Ca-EDTA (124.51 ± 18.91 pA, $n = 34$, $P > 0.05$ compared with controls; Fig. 2*A*). Considering that the K_d of EDTA for Zn^{2+} is 10^{16} (Bers 1982; Vogt et al. 2000), these concentrations of Ca-EDTA are 2 orders of magnitude higher than the apparent affinity constant of Ca-EDTA for Zn^{2+} (3–4 nM). On a practical note, further attempts to increase the concentration of Ca-EDTA (e.g., to 10 mM) (as in Huang et al. 2008; Li et al. 2001) yielded very unstable whole cell recordings, likely due to excessive buffering of divalent cations that reduced membrane charge screening. Screening and binding of negative charges on the extracellular surface of plasma membranes by divalent cations contributes to the electric field across the membrane, and thus is critical for normal membrane excitability (Hille 2001); in fact, Zn^{2+} itself exerts very potent charge screening effects (Blaustein and Goldman 1968; Hille et al. 1975).

Consistent with the lack of effect of extracellular Zn^{2+} on MF-evoked responses, membrane currents activated by application of recombinant BDNF to apical dendrites of CA3 pyramidal neurons (in the presence of TTX) were not significantly affected by the addition of 2.5 mM Ca-EDTA (control I_{BDNF} 83.67 ± 19.37 pA, $n = 27$, vs. I_{BDNF} in Ca-EDTA 79.61 ± 19.67 pA, $n = 16$; $P > 0.05$; Fig. 2*B*). We also tested whether I_{BDNF} was affected by increasing extracellular Zn^{2+} . I_{BDNF} evoked in the presence of 200 μ M $ZnCl_2$ was not

significantly different from controls (84.19 ± 17.01 pA, $n = 11$, vs. 83.67 ± 19.37 pA, $n = 27$; $P > 0.05$; Fig. 2*B*). Because recombinant BDNF applied to CA3 s. lucidum did not increase mEPSC frequency in CA3 pyramidal neurons (M. D. Amaral, Y. Li, T. Inoue, G. Calfa, L. Pozzo-Miller, unpublished data), these observations rule out potential artifacts arising from activity-dependent release of vesicular Zn^{2+} from MFs. Taken together, these results demonstrate that MF- I_{BDNF} is not sensitive to changes in the concentration of extracellular Zn^{2+} .

TRPC3 channels are required for MF- I_{BDNF}

To directly demonstrate the requirement for TRPC3 channels in the activation of MF- I_{BDNF} as we did for recombinant BDNF-induced responses in CA1 (Amaral and Pozzo-Miller 2007a), we used a shRNA that was specifically designed to knockdown TRPC3 channel expression. HuSH constructs encoding TRPC3 shRNA (and GFP used for cell identification) were coated onto gold particles and then biolistically transfected into slice cultures (Alonso et al. 2004; Lo et al. 1994). After 48 h of transfection, GFP or eYFP-expressing CA3 pyramidal neurons were identified by epifluorescence microscopy and the presence of a gold particle (Alonso et al. 2004; Amaral and Pozzo-Miller 2007a).

Consistent with our observations in CA1 neurons, TRPC3 knockdown completely prevented the activation of MF- I_{BDNF} in CA3 pyramidal neurons (27.63 ± 2.92 pA, $n = 42$, vs. untransfected CA3 neurons within the same slice 316.3 ± 41

pA, $n = 12$; $P < 0.001$; Fig. 2C). However, CA3 neurons transfected with a cDNA plasmid encoding eYFP alone or a random shRNA sequence did not affect MF- I_{BDNF} amplitude (311.88 ± 33.34 pA, $n = 14$; $P > 0.05$ compared with untransfected CA3 neurons in the same slice; Fig. 2C). These results demonstrate that MF- I_{BDNF} in CA3 neurons is mediated by nonselective cationic channels containing TRPC3 subunits.

Activity-dependent release of BDNF from MFs evokes dendritic Ca^{2+} signals in voltage clamped CA3 pyramidal neurons

Elevations in the intracellular concentration of Ca^{2+} ions were the first-described immediate actions of BDNF (Berninger et al. 1993). Activated neurotrophin Trk receptors trigger the formation of IP_3 by PLC γ -mediated hydrolysis of phosphatidylinositol biphosphate (PIP_2), which then activates IP_3 receptors of intracellular Ca^{2+} stores (Segal and Greenberg 1996). Consistently, I_{BDNF} evoked by recombinant BDNF in hippocampal CA1 neurons is associated with dendritic transient Ca^{2+} elevations (Amaral and Pozzo-Miller 2007b). Here we performed simultaneous whole cell recording and microfluorometric imaging in CA3 pyramidal neurons filled with the Ca^{2+} indicator fura-2 (100 μM) and voltage clamped at +40 mV to reduce the contribution of voltage-gated Ca^{2+} channels (Perkel et al. 1993; Pozzo-Miller et al. 1996). In the presence of our antagonist cocktail (which also included 20 μM of the L-type Ca^{2+} channels blocker nimodipine), TBS of MFs within CA3 s. lucidum evoked outward membrane currents followed by slowly developing Ca^{2+} elevations in apical dendrites (Fig. 3A, left). The 360/380 nm ratio of fura-2 signals had average peak amplitudes of 0.6 ± 0.06 ($n = 33$, CV = 0.54; Fig. 3B). To determine whether these Ca^{2+} responses were a result of activity-dependent BDNF release, afferent MFs were stimulated after 30 min incubation with the extracellular BDNF scavenger TrkB-IgG (Fig. 3A, right). Under these conditions, there were no significant changes in the fura-2 ratio (0.05 ± 0.01 , $n = 14$, CV = 0.93) when compared with control responses ($P < 0.001$; Fig. 3B).

TBS of MFs causes a BDNF-dependent depression of excitatory postsynaptic currents in CA3 pyramidal neurons

Considering the well-established role of BDNF on hippocampal synaptic plasticity (Bramham and Messaoudi 2005; Poo 2001), we monitored the amplitude of evoked EPSCs recorded from CA3 pyramidal neurons before and after a single brief TBS of afferent MFs. Pharmacologically isolated EPSCs mediated by AMPAR were significantly reduced after TBS of MFs (144.5 ± 26.8 pA vs. 38.52 ± 14.3 pA after 40 min, $n = 12$; $P < 0.001$; Fig. 4A); these responses were subsequently blocked by the AMPAR antagonist CNQX (20 μM). Similarly, pharmacologically isolated EPSCs mediated by NMDAR were significantly reduced after TBS of MFs (188.6 ± 13.5 vs. 79.8 ± 10.0 pA after 40 min, $n = 30$; $P < 0.001$; Fig. 4B); these responses were subsequently blocked by the NMDAR antagonist D,L-APV (50 μM). This enduring depression of NMDAR-mediated responses unlikely reflects the much short-lived Ca^{2+} -dependent inactivation of NMDARs (Legendre et al. 1993) because it developed even if the monitoring of NMDAR-mediated EPSCs was resumed after a 5 min interval from the theta burst stimulus, long enough for the TBS-induced Ca^{2+} transient to be dissipated by buffering, sequestration, and extrusion. Note that TBS of MFs also induced a brief posttetanic potentiation (PTP), which is evident when afferent stimulation was resumed immediately after TBS (see Supplemental Fig. S1B).

To obtain the ratio of NMDAR/AMPA EPSCs in the same neuron, 40–50 consecutive synaptic responses in control ACSF were first evoked at $V_{\text{hold}} = -60$ mV for AMPAR-EPSCs and then another EPSC series at +40 mV for NMDAR-EPSCs. Consistent with the pharmacological approach in different cells, the amplitude of AMPAR-EPSCs and of NMDAR-EPSCs were also significantly reduced after MF TBS (94.4 ± 7.6 vs. 55.7 ± 5.1 pA after 20 min, $n = 4$; $P < 0.001$ for AMPAR-EPSCs; 176.2 ± 25.2 vs. 122.5 ± 11.4 pA after 20 min, $n = 4$; $P < 0.05$ for NMDAR-EPSCs), while the NMDAR/AMPA ratio was unchanged (0.5 ± 0.06 vs. $0.4 \pm$

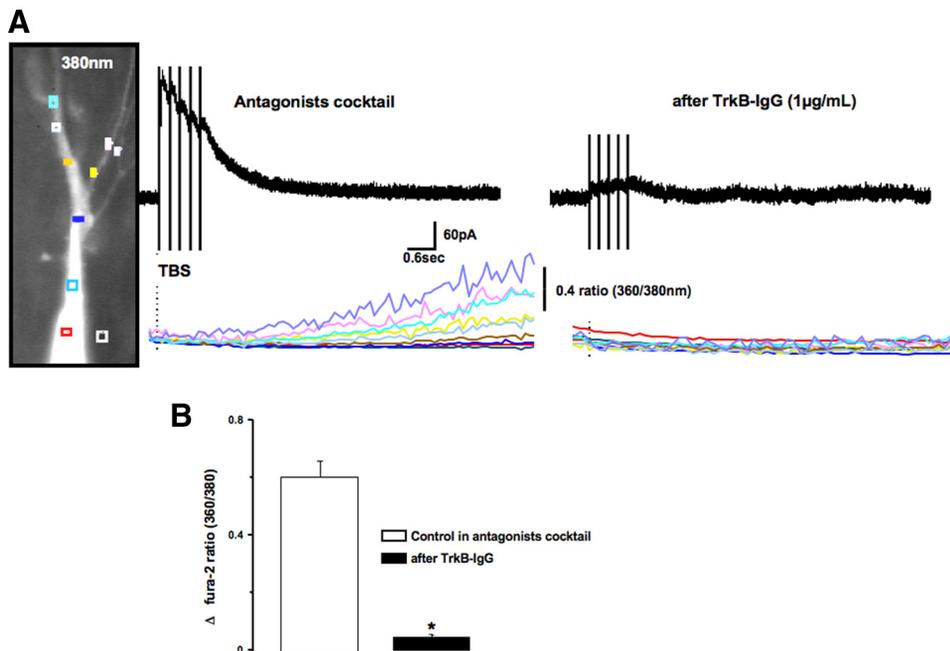


FIG. 3. MF- I_{BDNF} is associated with elevations in dendritic Ca^{2+} levels. *A, left*: representative example of Ca^{2+} signals evoked during MF stimulation in the presence of TTX, iono- and metabotropic glutamate, and GABA receptor antagonists. Ca^{2+} elevations occurred throughout the apical dendrites and the soma. *(right)*, The BDNF scavenger TrkB-IgG (1 $\mu\text{g}/\text{ml}$) prevented Ca^{2+} elevations during MF stimulation in the antagonist cocktail. *B*: quantification of the changes in peak fura-2 ratios (360 nm/380 nm) during MF stimulation.

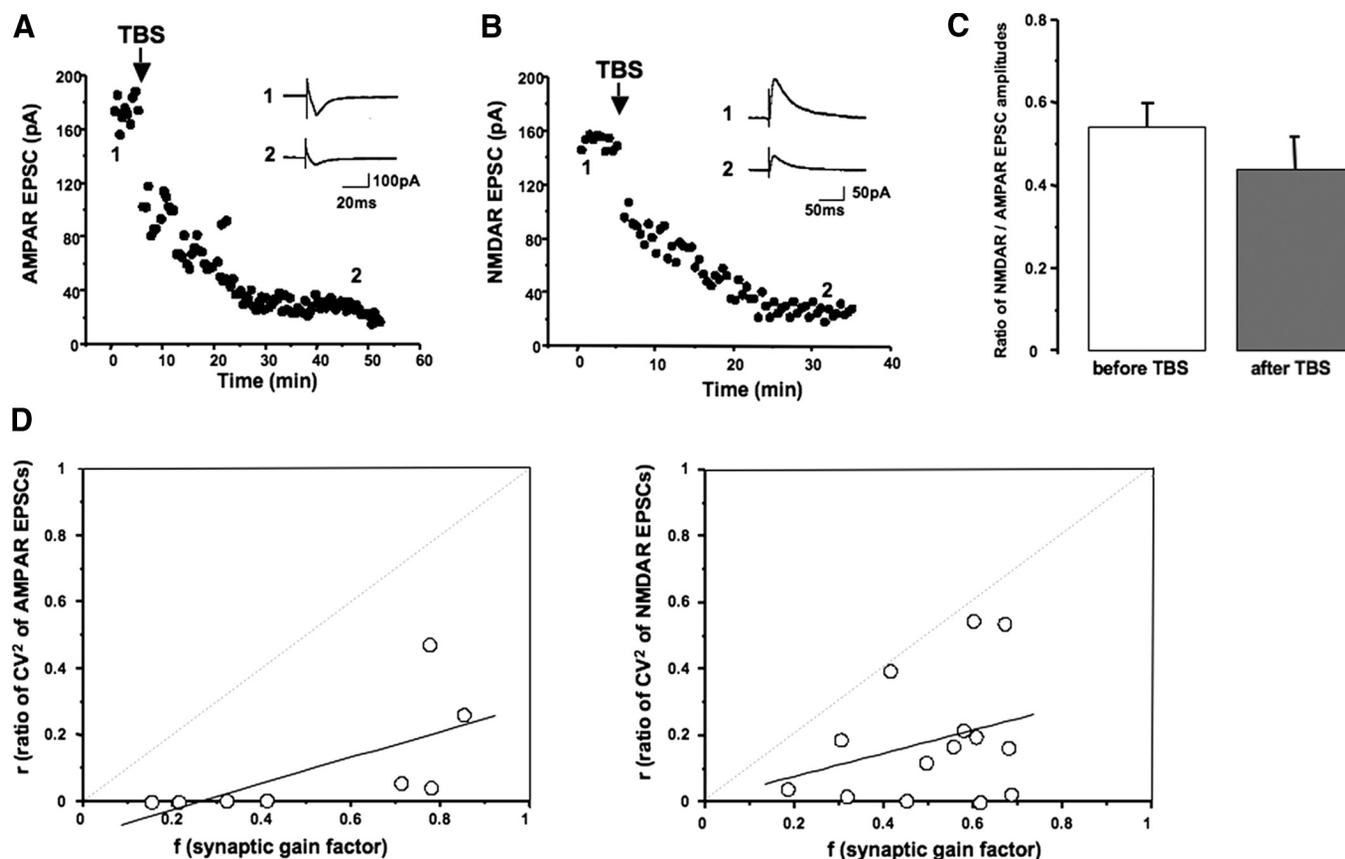


FIG. 4. A single brief TBS of MFs causes a BDNF-dependent depression of excitatory postsynaptic currents in CA3 pyramidal neurons. *A*: representative example illustrating the enduring depression of AMPAR-mediated EPSCs after TBS of MFs. *Inset*: representative EPSCs before (1) and 50 min after (2) TBS. *B*: representative example illustrating the enduring depression of *N*-methyl-D-aspartate receptor (NMDAR)-mediated EPSCs after TBS of MFs. *Inset*: representative EPSCs before (1) and 40 min after (2) TBS. *C*: TBS of MFs did not affect the NMDAR/AMPA ratio. *D*: plots of the fractional changes in coefficient of variation (CV^2 , r) vs. the synaptic gain factor (f) after TBS of MFs. Note that all points fall to the right of the diagonal ($r = f$).

0.08 after 30 min, $n = 5$; $P = 0.3$; Fig. 4C). Such parallel changes in the amplitude of both AMPAR-EPSCs and NMDAR-EPSCs with a constant NMDAR/AMPA ratio strongly suggest a presynaptic expression mechanism.

Changes in presynaptic function are usually accompanied by changes in the coefficient of variation (CV^2) of EPSC amplitudes. Consistent with a decrease in presynaptic transmitter release (e.g., Choi and Lovinger 1997; Shen and Horn 1996), the CV^2 of pharmacologically isolated AMPAR-EPSCs was significantly increased after TBS of MFs ($n = 8$, $P = 0.0039$ Wilcoxon signed-rank test); a similar increase was observed for the CV^2 of pharmacologically isolated NMDAR-EPSCs after MF TBS ($n = 14$, $P < 0.0001$ Wilcoxon signed-rank test). Also consistent with a presynaptic change (Faber and Korn 1991; Shen and Horn 1996), the plot of the fractional change in CV^2 (r) versus the synaptic gain factor (f) showed a significant correlation with all points to the right of the diagonal ($r = f$; AMPAR-EPSCs linear regression slope = 0.40, significantly different from unity $P = 0.0246$, $n = 8$; NMDAR-EPSCs linear regression slope = 0.35, significantly different from unity $P = 0.0012$, $n = 14$; Fig. 4D).

The role of activity-dependent release of endogenous BDNF on this enduring depression of NMDAR-mediated responses was tested using the scavenger TrkB-IgG. Indeed, the amplitude of NMDAR-mediated EPSCs remained unaffected after TBS in the presence of 1 μ g/ml of TrkB-IgG in the recording ACSF (165.7 ± 12.8 vs. 142.3 ± 15.3 pA after 20 min, $n = 9$;

$P = 0.29$; Fig. 5A). Consistent with a requirement of TrkB receptors, intracellular application of the membrane impermeable analog k-252b (200 nM in 0.01% DMSO) (Amaral and Pozzo-Miller 2007a; Knusel and Hefti 1992) also prevented TBS-induced depression of NMDAR-mediated EPSCs (185 ± 9.7 vs. 168.9 ± 7 pA after 20 min, $n = 13$; $P = 0.1763$; Fig. 5B). These observations suggest that an enduring depression of glutamatergic synaptic transmission at MF-CA3 synapses is a physiological consequence of activity-dependent release of endogenous BDNF from MFs.

DISCUSSION

We describe here several novel insights into the immediate actions of endogenous—native—BDNF on CA3 hippocampal neurons and of its activity-dependent release on excitatory synaptic transmission at MF-CA3 synapses. First, CA3 pyramidal neurons express I_{BDNF} , a nonselective cationic current evoked by TBS of afferent MFs that required TrkB receptors and TRPC3-containing ion channels. Consistent with such activity-dependent release of BDNF, these MF- I_{BDNF} responses were insensitive to manipulations of extracellular Zn^{2+} concentration. Second, MF- I_{BDNF} was accompanied by dendritic Ca^{2+} elevations, which were sensitive to the BDNF scavenger TrkB-IgG. Last, TBS of MFs induced a long-lasting depression in the amplitude of EPSCs mediated by both AMPA and NMDA receptors that was associated with changes in the

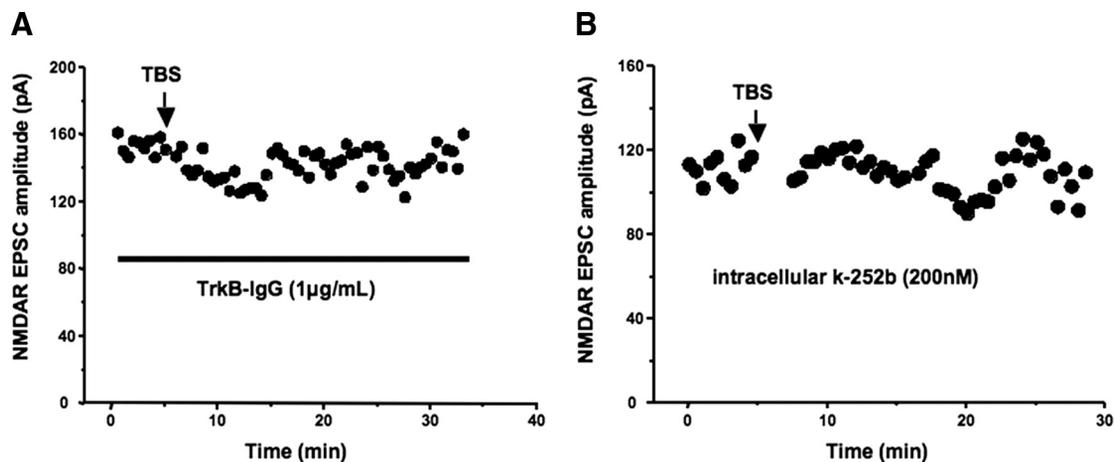


FIG. 5. The enduring depression of NMDAR-mediated EPSCs was sensitive to the BDNF scavenger TrkB-IgG and the receptor tyrosine kinase inhibitor k-252b. *A*: representative example illustrating the prevention of MF-TBS-induced depression of NMDAR-mediated EPSCs by bath application of TrkB-IgG. *B*: representative example illustrating the prevention of MF-TBS-induced depression of NMDAR-mediated EPSCs by intracellular application of k-252b.

coefficient of variation (CV^2) but not in the NMDAR/AMPA ratio, suggesting a presynaptic expression mechanism. Such enduring depression of NMDAR-mediated EPSCs required activity-dependent release of endogenous BDNF from MFs and activation of Trk receptors, as it was sensitive to TrkB-IgG and the tyrosine kinase inhibitor k-252b.

In the present studies we focused on the MF pathway in CA3 because these fibers contain the highest levels of BDNF in the brain (Conner et al. 1997; Danzer and McNamara 2004). We stimulated these afferent fibers with theta burst patterns because they were shown to be very effective at causing BDNF release (Balkowiec and Katz 2002; Hartmann et al. 2001). The inhibition of these membrane currents by the well-known BDNF scavenger TrkB-IgG (Shelton et al. 1995) but not by a control IgG confirmed that they reflected the activity-dependent release of endogenous BDNF. The fact that TrkB-IgG completely abolished these responses also suggests that the release of vesicular Zn^{2+} from MFs during TBS did not contribute to the activation of TrkB-initiated, TRPC-mediated membrane currents. This interpretation is also supported by the lack of effect of the membrane-impermeant Zn^{2+} chelator Ca-EDTA on responses evoked by either endogenous (i.e., MF- I_{BDNF}) or exogenous (i.e., recombinant) BDNF. Furthermore, increasing extracellular Zn^{2+} also failed to affect membrane currents evoked by direct application of recombinant BDNF. What role vesicular Zn^{2+} ions play is a matter of intense debate (Frederickson et al. 2005; Kay 2006; Kay and Toth 2008), one that is beyond the main topic of this communication. What is clear from our experiments is that trans-activation of TrkB receptors by synaptically released Zn^{2+} from MFs (Huang et al. 2008) does not contribute to the activation of TRPC3-mediated currents in CA3 pyramidal neurons.

Activation of a membrane conductance mediated by TRPC channels through the activation of the PLC γ pathway downstream of TrkB receptors seems to be the most immediate action of BDNF in central neurons. Support for this interpretation comes from observations of TRPC currents evoked within tens of seconds by exogenously applied recombinant BDNF in pontine neurons (Li et al. 1999) and CA1 pyramidal neurons (Amaral and Pozzo-Miller 2007a). Albeit with faster activation kinetics, membrane currents evoked by endogenously released BDNF are also sensitive to TrkB and TRPC

pharmacological inhibitors (Amaral and Pozzo-Miller 2007a) as well as to shRNA-mediated TRPC3 channel knockdown (Fig. 2A). Similar kinetic differences between TRPC currents evoked by group-I mGluR agonists and endogenously released glutamate have been observed in Purkinje cells (Kim et al. 2003), dopamine neurons of the substantia nigra compacta (Bengtson et al. 2004), and neurons in the lateral amygdala (Faber et al. 2006). The parsimonious interpretation is that the faster activation and smaller amplitude of MF- I_{BDNF} compared with I_{BDNF} reflects spatially and temporally restricted release of a few BDNF granules from MF boutons.

What is the physiological consequence of endogenous BDNF released from MFs onto CA3 pyramidal neurons? The theta burst patterns of MF stimulation that evoked MF- I_{BDNF} in CA3 neurons caused an enduring change in the strength of transmission at MF-CA3 synapses. Indeed, both AMPAR- and NMDAR-mediated EPSCs were significantly depressed after TBS of MFs, an effect prevented by the BDNF scavenger TrkB-IgG and the tyrosine kinase inhibitor k-252b. Because this depression was associated with changes in CV^2 but not in the NMDAR/AMPA ratio, its expression mechanism appears to be a reduction in presynaptic neurotransmitter release (Faber and Korn 1991; Shen and Horn 1996), an unexpected result considering the well-documented enhancement of presynaptic transmitter release by BDNF (Carvalho et al. 2008; Tyler et al. 2002). Regarding the LTP induced in CA3 pyramidal neurons by TBS of MFs reported by Nicholls et al. (2006), we should note that differences in the duration of TBS conditioning stimuli likely account for the different outcomes in that study and in the present communication. Despite the varied mechanisms and loci of induction of bidirectional synaptic plasticity at MF-CA3 synapses, most studies agree on a presynaptic site of expression (Nicoll and Schmitz 2005). For example, MF-LTP of AMPAR-mediated EPSCs is due to enhanced neurotransmitter release by cAMP/PKA-mediated modulation of synaptic vesicle docking and fusion, likely via a GTP-dependent interaction between Rab3A on synaptic vesicles and RIM1 α at the active zone (Castillo et al. 1997, 2002), although the direct phosphorylation of RIM1 α by PKA is not necessary for presynaptic LTP (Kaeser et al. 2008); it should be noted that MF-LTP of NMDAR-mediated responses is induced and expressed by entirely postsynaptic mechanisms (Kwon and Castillo 2008).

Relevant to the enduring depression of synaptic transmission described here, MF-CA3 synapses during the second postnatal week express two forms of NMDAR-independent LTD that differ in their dependency on mGluR activation (Domenici et al. 1998). However, both of these forms of MF-LTD are induced by prolonged low-frequency stimulation, which is not sufficient to cause BDNF release (e.g., Balkowiec and Katz 2002; Hartmann et al. 2001). MF-CA3 synapses also express a presynaptic form of long-term depression (LTD) of AMPAR-mediated EPSCs (Kobayashi et al. 1996) that involves presynaptic cAMP/PKA signaling (Tzounopoulos et al. 1998), indicating that the above-described presynaptic modulation of synaptic vesicle docking and fusion is bidirectional. Intriguingly, BDNF/TrkB signaling may also involve the active zone protein RIM1 α , as the presynaptic effect of BDNF is absent in cultured hippocampal neurons lacking its activator Rab3A in synaptic vesicles (Alder et al. 2005; Thakker-Varia et al. 2001). Because most studies of the presynaptic effects of BDNF were performed either in dissociated cultures of hippocampal neurons (Jovanovic et al. 1996, 2000; Yano et al. 2006)—which almost exclusively contain pyramidal neurons and lack dentate granule cells and their MF boutons—or at CA3-CA1 synapses in hippocampal slices (Figurov et al. 1996; Gottschalk et al. 1998; Pozzo-Miller et al. 1999; Tyler et al. 2006), it remains to be determined whether MF boutons have different intracellular signaling cascades or synaptic vesicle and/or active zone molecular targets than their Schaffer collateral counterparts.

In summary, we uncovered the most immediate response to endogenously released—native—BDNF in hippocampal neurons and its role in the modulation of excitatory synaptic strength at MF-CA3 synapses, lending further credence to the relevance of BDNF signaling for synaptic function in the hippocampus.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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