Development/Plasticity/Repair

Activation of Adenosine A_{2A} Receptor Facilitates Brain-Derived Neurotrophic Factor Modulation of Synaptic Transmission in Hippocampal Slices

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Both brain-derived neurotrophic factor (BDNF) and adenosine influence neuronal plasticity. We now investigated how adenosine influences the action of BDNF on synaptic transmission in the CA1 area of the rat hippocampal slices. Alone, BDNF (20–100 ng/ml) did not significantly affect field EPSPs (fEPSPs). However, a 2 min pulse of high-K^+ (10 mM) 46 min before the application of BDNF (20 ng/ml) triggered an excitatory action, an effect blocked by the TrkB receptor inhibitor K252a (200 nM), by the adenosine A_{2A} receptor antagonist ZM 241385 (50 nM), and by the protein kinase A inhibitor H-89 (1 μM). Presynaptic, rather than postsynaptic depolarization was required to trigger the BDNF action because after K^+ depolarization BDNF also increased EPSCs recorded from pyramidal neurons voltage-clamped at −60 mV, and transient postsynaptic depolarization was unable to unmask the BDNF action. A weak theta burst stimulation of the afferents could elicit potentiation of synaptic transmission only when applied in the presence of BDNF. Activation of adenosine A_{2A} receptors with CGS 21680 (10 nM), or the increase in extracellular adenosine levels induced by 5-iodotubercidin (100 nM) triggered the excitatory action of BDNF, a process prevented by ZM 241385 and by H-89. In the presence of dibutyryl-cAMP (0.5 mM), BDNF also increased fEPSPs but postsynaptic cAMP (0.5 mM) was unable to trigger the BDNF action.

It is concluded that presynaptic activity-dependent release of adenosine, through activation of A_{2A} receptors, facilitates BDNF modulation of synaptic transmission at hippocampal synapses.

Key words: **adenosine receptors; BDNF; hippocampus; synaptic transmission; neuromodulation; neurotrophic factors**

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, that in mammals includes nerve growth factor, neurotrophin-3, and neurotrophin-4/5. These belong to a group of signaling factors that are essential for the regulation of neuronal survival, differentiation, and cell death events (Lewin and Barde, 1996). Besides these long-lasting actions, BDNF has presynaptic regulatory actions on synaptic transmission, inducing an increase in the number of docked vesicles at the active zones (Tyler and Pozzo-Miller, 2001), as well as postsynaptic regulatory actions (Levine el at., 1998). BDNF also enhances and/or induces long-term potentiation (LTP) either *in vitro* (Kang and Schuman, 1995; Figurov et al., 1996; Kovalchuk et al., 2002) or *in vivo* (Messaoudi et al., 2002). Most of these actions have been seen to occur in the hippocampus, which is a prominent site of expression of BDNF and of its specific tyrosine kinase receptor (TrkB) (Klein et al., 1990).

The hippocampus is under neuromodulatory control by adenosine, which through activation of inhibitory (A_1) and excitatory (A_{2A}) receptors fine tunes the action of neurotransmitters and neuromodulators (Sebastião and Ribeiro, 2000). Recently, through immunoprecipitation and immunoblotting methods, it was observed that adenosine and adenosine agonists can induce TrkB phosphorylation through a mechanism involving the adenosine A_{2A} receptor (Lee and Chao, 2001). These data together with the finding that adenosine A_{2A} receptors and neurotrophin receptors have a considerable overlap in their distribution (Lewin and Barde, 1996; Fredholm et al., 2001), prompted us to evaluate how endogenous activation of adenosine A_{2A} receptor could influence the action of BDNF on hippocampal synaptic transmission. Because the excitatory action of BDNF on synaptic transmission at the developing neuromuscular junction is facilitated by depolarization (Boulanger and Poo, 1999a) and depolarizing conditions are able to induce adenosine release (Pazzagli et al., 1993), we also investigated how depolarizing conditions influence BDNF actions and how this could be related to adenosine receptor activation.

We found that the excitatory action of BDNF on synaptic transmission in the hippocampus can be induced by a presynaptic depolarization and is dependent on adenosine A_{2A} receptor

Received June 2, 2003; revised Jan. 20, 2004; accepted Jan. 20, 2004.

This work, M.J.D., and C.C.F. were supported by Fundação para a Ciência e Tecnologia. We thank Regeneron for the gift of brain-derived neurotrophic factor and Dr. W. W. Andersen (University of Bristol, Bristol, UK) for the kind gift of the data analysis (LTP) program. The animal housing facilities of the Institute of Physiology of the Faculty of Medicine of Lisbon are also acknowledged.

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DOI:10.1523/JNEUROSCI.4454-03.2004

 0270-6474/04/242905-09$15.00/0

activation, through a mechanism that requires cAMP formation and protein kinase A (PKA) activity.

Materials and Methods

Preparation of hippocampal slices. The experiments were performed on hippocampal slices preparations from male Wistar rats (3–4 weeks old) from Harlan Interfauna Iberica, SL (Barcelona). The animals were handled according to European Community guidelines and Portuguese law on Animal Care and anesthetized with halothane before decapitation.

Field EPSP recordings. The hippocampus was dissected free within ice-cold Krebs' solution composed of (mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400-μm-thick) were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for at least 1 hr in a resting chamber, filled with the same solution, at room temperature (22–25°C). The slices were transferred to a recording chamber (1 ml plus 6 ml dead volume) for submerged slices and continuously superfused at 3 ml/min with gassed bathing solution at 32°C; the drugs were added to this superfusion solution. Field EPSPs (fEPSPs) were recorded as previously in our laboratory (Sebastião et al., 2001) through an extracellular microelectrode (4 M NaCl, 2–6 MΩ resistance) placed in the stratum radiatum of the CA1 area. Stimulation (rectangular 0.1 msec pulses, once every 15 sec) was delivered through a concentric electrode placed on the Schaffer collateral-commissural fibbers, in the stratum radiatum near the CA3–CA1 border. The intensity of stimulus (80–200 μA) was initially adjusted to obtain a large fEPSP slope with a minimum population spike contamination. Recordings were obtained with an Axoclamp 2B amplifier and digitized (Axon Instruments, Foster City, CA). Individual responses were monitored, and averages of eight consecutive responses were continuously stored on a personal computer with the LTP program (Anderson and Collingridge, 2001).

EPSC recordings. The brain was removed, hemisected, and trimmed to contain a block of tissue surrounding the hippocampus. Transverse slices (300-μm-thick) were cut on a vibratome (VT 1000 S; Leica, Nussloch, Germany) in ice-cold dissecting solution containing (mM): sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 1; $NaHCO_3$ 25; NaH_2PO_4 1.25; glucose 10, ascorbate 3; and pyruvic acid sodium salt 1.3, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. Slices were preincubated in Krebs' solution containing (mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and glucose 10, gassed with a 95% O_2 and 5% CO_2, pH 7.4, at 35°C for 30 min and used at least 1 hr after recovering at room temperature. Individual slices were fixed on a grid in a recording chamber (1 ml plus 200 μl dead volume) for submerged slices and continuously superfused at 3 ml/min with Krebs' solution at room temperature; the drugs were added to this superfusion solution. Patch electrodes (5–10 MΩ) were filled with internal solution containing (mM): potassium gluconate 125; KCl 11; $CaCl_2$ 0.1; $MgCl_2$ 2; EGTA 1; HEPES 10; NaATP 2; NaGTP 0.3; and QX-314 5 (to intracellularly block voltage-dependent sodium channels), pH 7.3, adjusted with KOH, 280–290 Osm. Stimulation (0.2 msec rectangular pulses once every 15 sec) was delivered as for fEPSP recordings. CA1 pyramidal cells were visually identified with a Carl Zeiss (Jena, Germany) Axioskop 2 FS upright microscope coupled to an IR-CCD camera. Whole-cell EPSCs were recorded in voltage-clamp mode ($V_h = -60$ mV) with an EPC-7 amplifier (List Biologic, Campbell, CA). Offset potentials were nulled directly before formation of a seal. Small voltage steps (5 mV, 50 msec) were evoked before an EPSC to monitor membrane and series resistances; if one of both or holding current changed significantly, the experiment was rejected. Junction potentials and voltage errors caused by series resistance were not corrected. The current signal was filtered using the 10 and 3 kHz three-pole Bessel filter of the EPC-7. Data acquisition was under the control of LTP software (Anderson and Collingridge, 2001). The averages of four consecutive individual recordings were obtained for analysis.

Drugs. BDNF was generously provided by Regeneron Pharmaceuticals (Tarrytown, NY). 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamido adenosine (CGS-21680), 5′-cAMP Tris salt, N^6,2′-*o*-dibutyryladenosine-3′:5′-cAMP (dbcAMP), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 5-iodotubercidin (ITU), *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89) were from Sigma (St. Louis, MO). 4-(2-[7-amino-2-(2-furyl)-[1,2,4]triazolo[2,3-a][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385) was from Tocris Cookson (Ballwin, MO). K-252a and 2-(triethylamino)-*N*-(2,6-dimethylphenyl)acetamide (QX-314) were obtained from Calbiochem (La Jolla, CA). BDNF was supplied in a 1.0 mg/ml stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer, and 0.004% Tween 20. DPCPX was made up in a 5 mM stock solution in 99% dimethylsulfoxide (DMSO) and 1 M 1% NaOH (v/v). CGS 21680, ZM 241385, and H-89 were made up in 5 mM stock solutions in DMSO. ITU was made up in a 10 mM stock solution in DMSO. K252a was made up in a 1 mM stock solution in DMSO. Aliquots of these stock solutions were kept frozen at −20°C until use.

Analysis of the data. The data are expressed as mean ± SEM from *n* number of slices. To allow comparisons between different experiments, the slope and amplitude values were normalized, taking as 100% of the averaged of the five values obtained immediately before applying the test compound. The significance of differences between the mean values obtained in test and control conditions was evaluated by Student's *t* test. Values of $p < 0.05$ were considered to represent statistically significant differences.

Results

Pre-depolarization induced by high K^+ facilitates BDNF excitatory action on hippocampal synaptic transmission through Trk receptors

As illustrated in Figure 1*A*, BDNF (20 ng/ml) when applied alone to the hippocampal slices did not significantly influence the slope of fEPSP ($n = 7$). Even at a higher concentration (100 ng/ml) BDNF could not enhance synaptic transmission ($n = 2$) (Fig. 1*B*). However, when applied to slices that had been shortly depolarized (Fig. 1*C*) with a pulse of high-K^+ (10 mM) for 2 min, 46 min before application of the neurotrophin, BDNF (20 ng/ml) caused a significant increase in fEPSP slope (41 ± 9.8% increase; $n = 9$; $p < 0.05$). The depolarizing pulse of high-K^+ (10 mM) for 2 min, caused a transient enhancement of fEPSP slope, which returned to the basal level, within 35 min after returning to the normal K^+ concentration in the bath; in slices where BDNF was not applied ($n = 3$), the fEPSP slope remained within those basal levels for at least 60 min.

To evaluate the type of receptor involved in the excitatory action of BDNF on synaptic transmission, we studied the effect of this neurotrophin in the presence of K252a, an inhibitor of Trk receptors phosphorylation (Berg et al., 1992). This inhibitor was added to the perfusion solution 30 min before the pulse of high-K^+ and remained in the bath up to the end of the experiment. The slices were therefore perfused with K252a (200 nM) for 76 min before BDNF application. K252a (200 nM) was virtually devoid of effect on synaptic transmission, and as shown in Figure 1*D*, it abolished the excitatory effect of BDNF ($n = 3$; $p < 0.05$).

Excitatory action of BDNF on synaptic responses does not depend on postsynaptic depolarization

To evaluate whether postsynaptic depolarization was required to trigger the excitatory actions of BDNF on synaptic transmission, whole-cell EPSCs were recorded from CA1 pyramidal neurons with membrane potential clamped at −60 mV throughout the experiment, including during the application of the high-K^+ depolarizing pulse (10 mM for 2 min).

Bath application of BDNF (20 ng/ml), 46 min after the high-K^+ pulse enhanced the peak amplitude of EPSCs (35 ± 7.3% increase; $n = 7$; $p < 0.05$). The maximal effect was already attained at ~15 min after beginning BDNF application and it lasted up to the end of the recording period (Fig. 2*A*). In slices that had not been exposed to the K^+-depolarizing pulse, BDNF

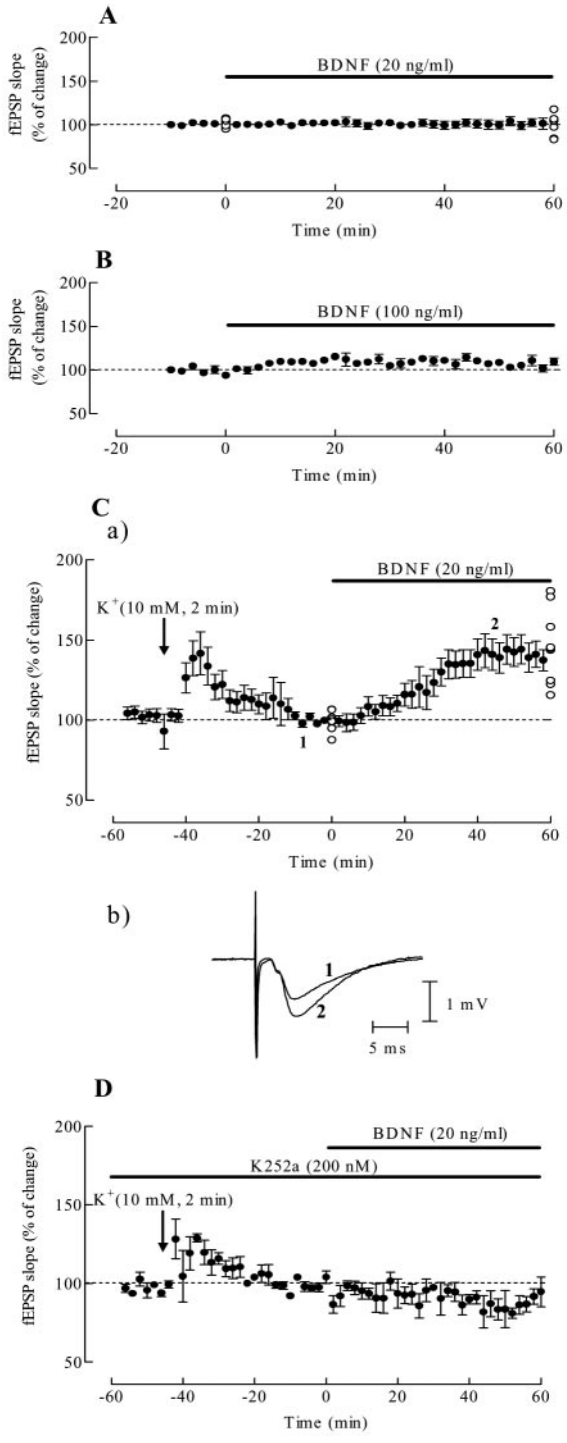

Figure 1. Predepolarization induced by high K^+ facilitates BDNF excitatory action on hippocampal synaptic transmission through Trk receptors. In *A* and *B* are shown the averaged time courses of changes in fEPSP slope induced by application of 20 ng/ml (corresponding to ~0.8 nM) (*A*), or 100 ng/ml (*B*) BDNF alone. *Ca* illustrates the averaged time course of changes in fEPSP slope caused by BDNF (20 ng/ml), which was perfused 46 min after treatment with high-K^+ (10 mM) for 2 min. *Cb* shows traces obtained in a representative experiment in *Ca*; each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. *D* shows the averaged time course of the effect of BDNF (20 ng/ml) applied 46 min after treatment with high-K^+ (10 mM) for 2 min in the presence of an inhibitor of TrkB receptors, K252a (200 nM), which was added to the slices 30 min before the pulse of high-K^+. Values obtained in individual experiments at time 0 and 60 min are shown as a scatter representation in *A* and *Ca*. In all panels, the arrows represent the beginning of the 2 min high-K^+ application, and the horizontal bars represent the application of the different drugs. All values

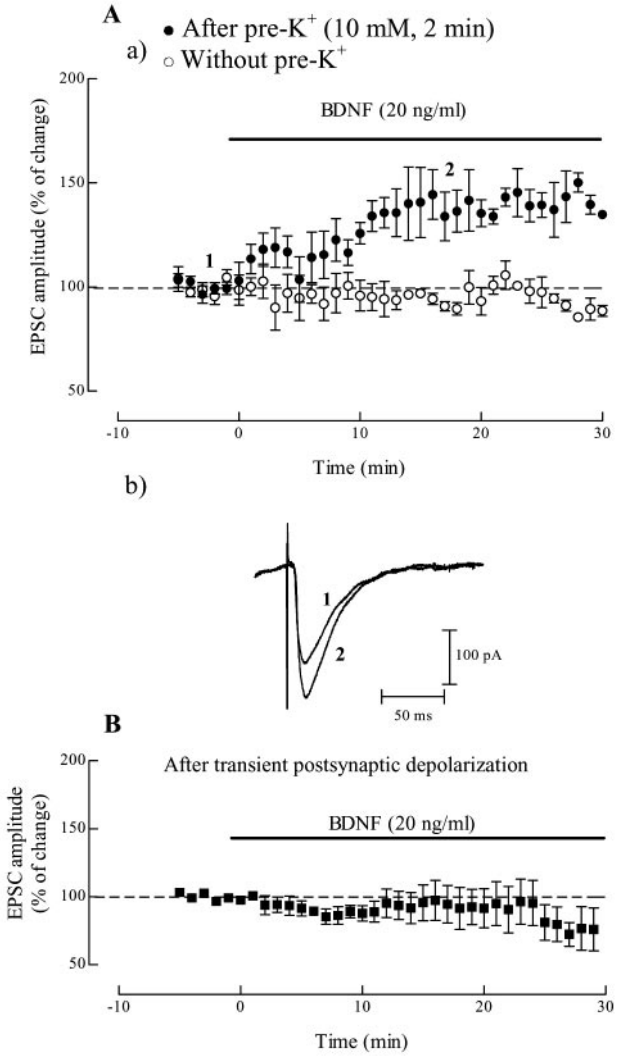

Figure 2. The excitatory action of BDNF on synaptic transmission does not depend on postsynaptic depolarization. *Aa* shows the averaged time course of changes in EPSC amplitude recorded from CA1 pyramidal cells under voltage clamp conditions ($V_h = -60$ mV). BDNF (20 ng/ml) was applied alone (○, without pre-K^+, $n = 3$) or 46 min after a depolarizing pulse of 10 mM KCl for 2 min (●, after pre-K^+, $n = 7$), and remained in the bath up to the end of the experiments, as indicated by the horizontal bar. *Ab* shows traces obtained in a representative experiment in Aa (●); each trace is the average of four consecutive responses obtained immediately before (1) and during (2) BDNF (20 ng/ml), and is composed of the stimulus artifact followed by the EPSC; resting membrane potential of this cell (measured in the current-clamp mode at the end of the experiment): −60 mV; input resistance: 180 MΩ. *B* shows the averaged time course of the effect of BDNF, which was added to the slices 46 min after transient depolarization of postsynaptic neurons by changing, for 6 min, the holding potential in the voltage-clamp mode from −60 to −40 mV and then again to −60 mV ($n = 4$). Values in the ordinates are mean ± SEM, in which 100% was taken as the averaged EPSCs amplitudes recorded for 5 min immediately before BDNF application (100% values ranged from −50 to −370 pA in the different experiments).

(20 ng/ml) was virtually devoid of effect on EPSC amplitude ($n = 3$) (Fig. 2*Aa*).

From the above results we could conclude that postsynaptic depolarization was not required to trigger the BDNF action. We

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are mean ± SEM; 100% (averaged fEPSP slopes at times −10–0): −0.64 ± 0.05 mV/msec, $n = 7$ (*A*), −0.70 ± 0.03 mV/msec, $n = 2$ (*B*), −0.57 ± 0.03 mV/msec, $n = 9$ (*C*), and −0.50 ± 0.10 mV/msec, $n = 3$ (*D*). Note that BDNF increased fEPSP slope only in predepolarized slices, an action prevented by the treatment with K252a.

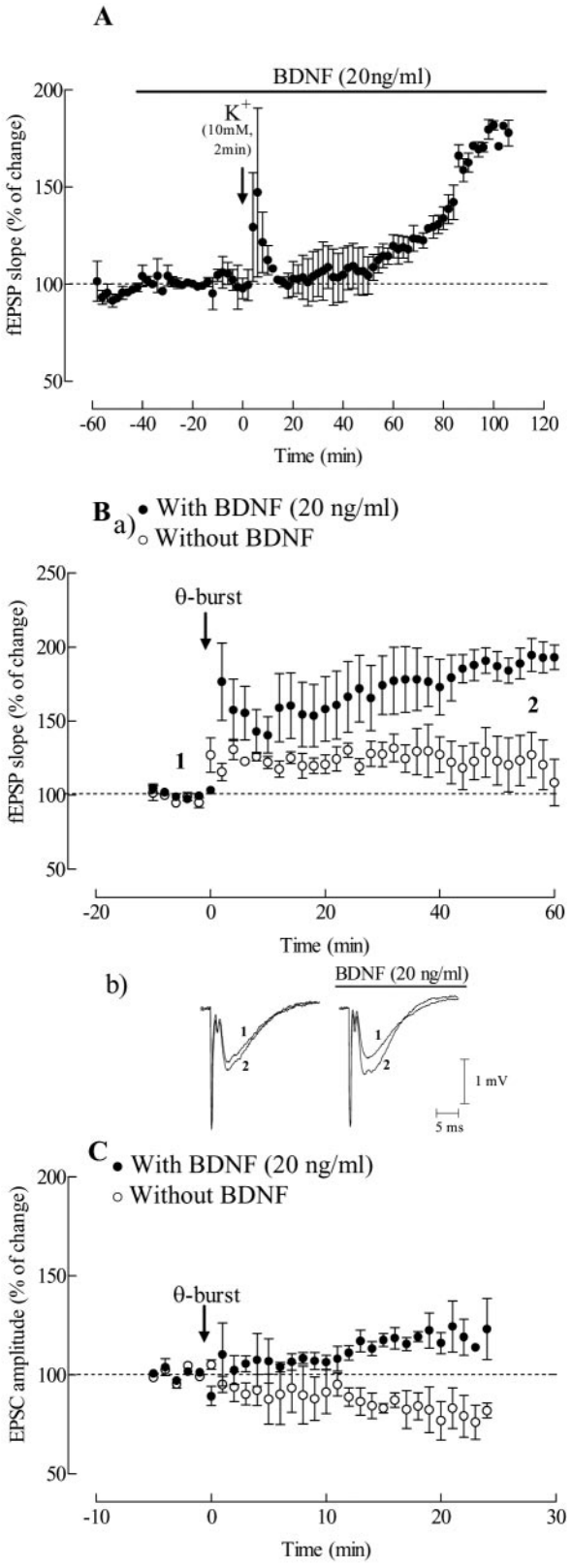

Figure 3. Presynaptic theta burst stimulation paired with BDNF can elicit synaptic potentiation. *A* shows the averaged time course of changes in fEPSP slope observed when the high-K^+ pulse was applied to slices in the presence of BDNF (20 ng/ml). *Ba* illustrates the averaged time course of changes in fEPSP slope observed after a weak theta burst stimulation (3 bursts at 5 Hz, each composed of three pulses at 100 Hz) in the presence of 20 ng/ml BDNF (●), which was added at least 10 min before electrical stimulation, as compared with the changes of fEPSP slope in the same conditions of stimulation but without administration of the neurotrophin (○). *Bb* shows traces obtained in representative experiments in *Ba*; each trace is the average of eight consecutive responses obtained immediately before (1) and after (2) theta burst stimulation in the absence (left) or in the presence (right) of BDNF, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP; recordings obtained in the same experiment are superimposed. *C* represents the averaged time course of changes in EPSC amplitude induced under voltage-clamp conditions ($V_h = -60$ mV) by theta burst stimulation in the presence (●) and in absence (○) of BDNF (20 ng/ml). In all panels the arrow represents the beginning of stimulation and the horizontal bar in *A* indicates the presence of BDNF. All values are mean ± SEM; 100% (averaged fEPSP slopes at times −10–0): −0.61 ± 0.07 mV/msec, $n = 2$ (*A*), −0.70 ± 0.02 mV/msec, $n = 3$ (*Ba*, ●), −0.66 ± 0.01 mV/msec, $n = 3$ (*Ba*, ○); 100% values in *C* ($n = 2$ for both ● and ○) represent EPSCs amplitudes recorded at times −5–0) and ranged from −110 to −550 pA.

next examined whether postsynaptic depolarization was sufficient to unmask the effect of BDNF. When recording in the current-clamp configuration it was observed that cells depolarized, as expected from the Nernst equation, from −60 to −40 mV during 6 min because of the pulse of high-K^+ (10 mM for 2 min) ($n = 2$). We therefore mimicked the action of the pulse of high-K^+ on postsynaptic cell by changing the holding potential in the voltage-clamp mode from −60 to −40 mV and then again to −60 mV, all during 6 min. BDNF (20 ng/ml) applied 46 min after this postsynaptic depolarization did not induce an increase in EPSC amplitude ($n = 4$) (Fig. 2*B*).

Presynaptic theta burst stimulation paired with BDNF can elicit synaptic potentiation

The K^+ depolarizing stimulus could still be effective if applied after BDNF administration. Indeed, when BDNF (20 ng/ml) was added to the slice at least 30 min before the K^+ depolarizing pulse (10 mM, 2 min) and remained in the bath up to the end of the experiment, an increase (81 ± 2.7%; $n = 2$) in fEPSP slope was observed (Fig. 3*A*), which is clearly different from what occurred when the BDNF (20 ng/ml) was applied without the K^+ pulse, or when the K^+ pulse was applied without BDNF (see above). Because the K^+ pulse causes a broad depolarization in all cells, including glial cells and interneurons, we evaluated whether a focal depolarization delivered through the stimulation electrode placed at the synaptic afferents (Schaffer collaterals) was able to elicit the BDNF effect. Weak theta burst stimulation (three bursts of three pulses each at 100 Hz, delivered 100 msec apart) was applied to slices that had been previously perfused with BDNF (20 ng/ml) for at least 10 min, and fEPSPs were increased by 91 ± 9.5% at 60 min after stimulus ($n = 3$, $p < 0.05$ as compared with values before stimulation) (Fig. 3*B*); as expected (de Mendonça and Ribeiro, 2000), the same stimulation applied in the absence of BDNF caused a smaller and non sustained increase in fEPSP slope (20 ± 16.1% increase 60 min after stimulation; $n = 3$; $p > 0.05$).

BDNF can facilitate LTP in particular under strong theta burst stimulation conditions (Chen et al., 1999). We next examined whether the facilitatory action of BDNF was also present in conditions in which LTP-like phenomena could not occur, i.e., when the postsynaptic membrane potential was clamped at −60 mV throughout the experiment, including during the theta burst stimulation of the Schaffer collaterals. Under these conditions, weak theta burst stimulation in the presence of BDNF (20 ng/ml) led to a gradual increase in the amplitude of EPSCs (19 ± 8.8; $n = 2$) (Fig. 3*C*), which is significantly different ($p < 0.05$) from what occurred in slices stimulated in the absence of BDNF, where synaptic currents even decreased (20 ± 8.6; $n = 2$).

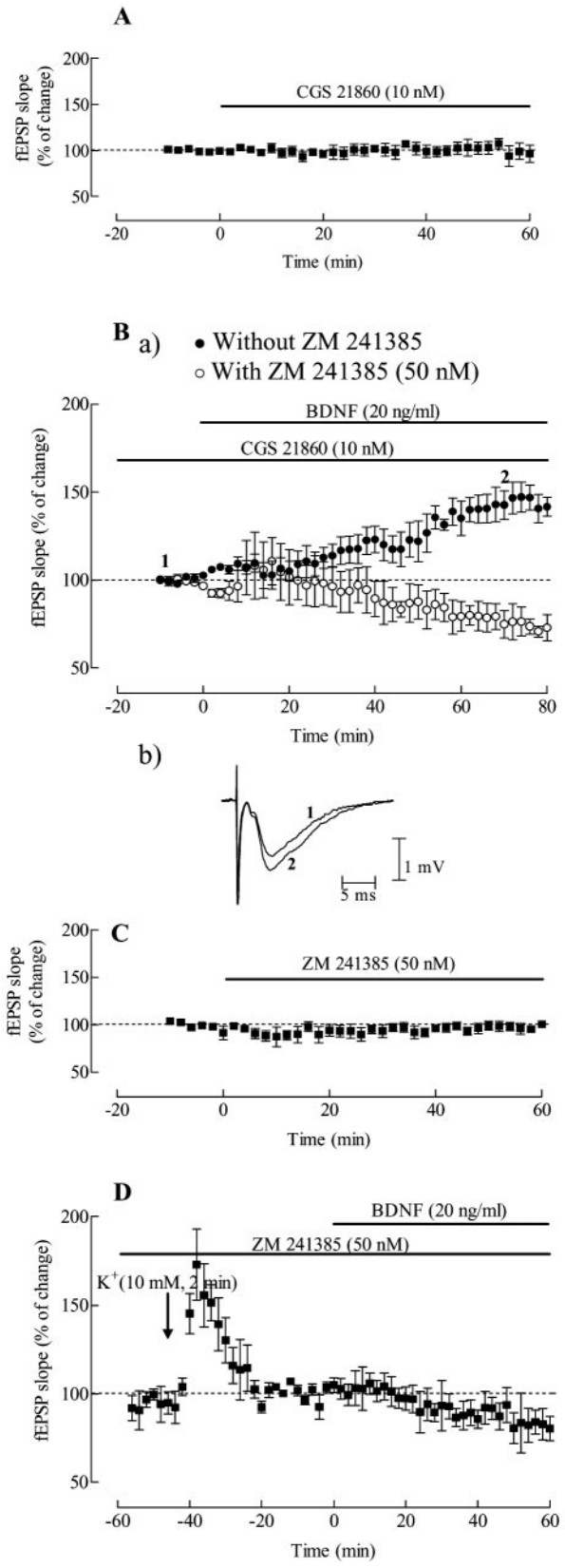

Figure 4. Adenosine A_{2A} receptors activation facilitates BDNF excitatory action on synaptic transmission. *A* shows the averaged time course of changes in fEPSP slope induced by application of 10 nM CGS 21860 alone. *Ba* shows the averaged time courses of the effect of BDNF (20 ng/ml) in the presence of the A_{2A} receptor agonist CGS 21680 (10 nM) (●) or in the presence of both CGS 21680 (10 nM) and the A_{2A} receptor antagonist ZM 241385 (50 nM) (○). CGS 21680 was applied at least 30 min before BDNF application, and ZM 241385 was applied 30 min before CGS 21680 application. *Bb* shows traces obtained in a representative experiment in Ba (●), each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. *C* shows the averaged time course of changes in fEPSP slope induced by application of 50 nM ZM 241385 alone. *D* shows the averaged time course of the effect of BDNF after a depolarizing stimulus of K^+ (10 mM) in the presence of ZM 241385; slices were superfused with BDNF (20 ng/ml) 46 min after treatment with high-K^+ (10 mM) for 2 min and were in the presence of ZM 241385 (50 nM), which was added for at least 30 min before the potassium pulse. In all panels the horizontal bars represent drug application, and the arrow in *D* represents the beginning of the 2 min high-K^+ application. All values are mean ± SEM; 100% (averaged fEPSP slopes at times −10–0): −0.60 ± 0.09 mV/msec, $n = 3$ (*A*), −0.61 ± 0.01 mV/msec, $n = 6$ (*Ba*, ●), −0.53 ± 0.14 mV/msec, $n = 3$ (*Bb*, ○), −0.54 ± 0.090 mV/msec, $n = 3$ (*C*), −0.58 ± 0.06 mV/msec, $n = 3$ (*D*). Note that activation of adenosine A_{2A} receptors facilitates the excitatory action of BDNF on synaptic transmission, an action prevented by A_{2A} receptor blockade.

Adenosine A_{2A} receptors activation facilitates BDNF excitatory action on synaptic transmission

To evaluate if adenosine A_{2A} receptors activation could influence the action of BDNF on synaptic transmission we first tested whether a selective agonist of the adenosine A_{2A} receptor CGS 21680 (Jarvis et al., 1989) affected BDNF action. CGS 21680 (10 nM) was added to the slices at least 30 min before BDNF application and was virtually devoid of effect on fEPSP slope (Fig. 4*A*). In the presence of CGS 21680 (10 nM), BDNF increased (Fig. 4*B*) the slope of fEPSPs by 43 ± 6.7% ($n = 6$; $p < 0.05$) in slices that had not been predepolarized by the high-K^+ pulse. Blockade of adenosine A_{2A} receptors with the selective antagonist (Poucher et al., 1995), ZM 241385 (50 nM), which was added 30 min before CGS 21680 (10 nM), prevented this excitatory effect of BDNF ($n = 3$; $p < 0.05$ as compared with absence of ZM 241385) (Fig. 4*B*). Indeed, in the presence of ZM 241385 (50 nM) plus BDNF (20 ng/ml) even decreased (26 ± 6.5%) rather than increased, fEPSP slope (Fig. 4*B*). ZM 241385 (50 nM) was virtually devoid of effect on fEPSP slope (Fig. 4*C*).

To know whether the adenosine A_{2A} receptor could play a role in the excitatory action of BDNF observed after K^+ depolarization, we studied how adenosine A_{2A} receptor blockade could influence that effect of BDNF. In the presence of the adenosine A_{2A} a receptor antagonist, ZM 241385 (50 nM), which was applied for at least 30 min before the K^+ pulse, the excitatory action of BDNF (20 ng/ml) was completely prevented ($n = 3$; $p < 0.05$) (Fig. 4*D*). Under these conditions BDNF (20 ng/ml) even decreased (17 ± 8.0%) fEPSP slope as it occurred in the presence of CGS 21680 (10 nM) plus ZM 241385 (50 nM).

The excitatory action of BDNF is facilitated by a selective adenosine kinase inhibitor 5-iodotubercidin

Our findings that a brief depolarization by a pulse of high-K^+ (10 mM) and subsequent application of BDNF resulted in a significant increase in fEPSP slope, together with previous findings that treatment of slices with high-K^+ Krebs' solution results in an increase of adenosine release (Pazzagli et al., 1993), prompted us to investigate if an enhancement of the extracellular adenosine levels could mimic the action of the pulse of high-K^+. We used a selective adenosine kinase inhibitor, 5-iodotubercidin (ITU), which enhances the concentration of endogenous extracellular adenosine at hippocampal slices (Pak et al., 1994). The application of ITU (100 nM) caused a decrease in synaptic transmission, which may be attributed to activation of adenosine A_1 receptors (the predominant adenosine receptors in the hippocampus) because it was prevented by the specific adenosine A_1 receptor antagonist (Lohse et al., 1987), DPCPX (50 nM) (Fig. 5*A*). In the

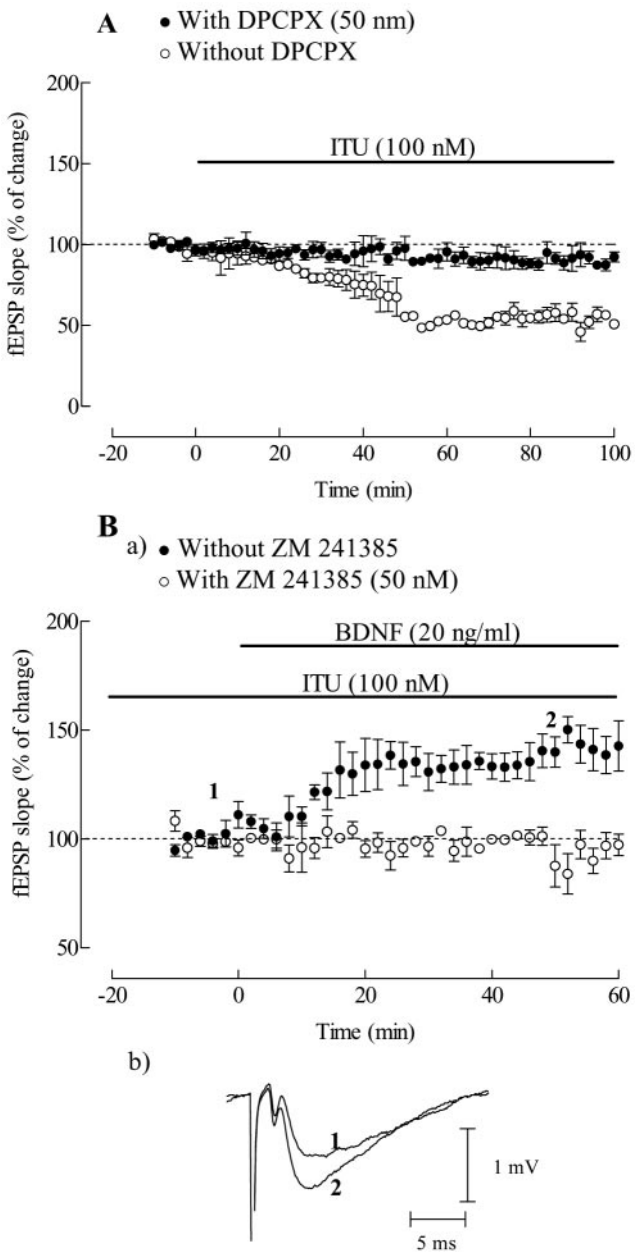

Figure 5. The excitatory action of BDNF is facilitated by a selective adenosine kinase inhibitor 5-iodotubercidin. *A* shows averaged time courses of changes of fEPSP slope in the presence of 5-iodotubercidin (ITU; 100 nM) (○) and in the presence of both ITU (100 nM) plus DPCPX (50 nM) (●), an A_1 adenosine receptor antagonist. *Ba* shows the averaged time courses of changes in fEPSP slope induced by BDNF (20 ng/ml) in the presence of ITU (100 nM), (●) or in the presence of both ITU (100 nM) plus the A_{2A} adenosine receptor antagonist, ZM 241985 (50 nM) (○). *Bb* shows traces obtained in a representative experiment in Ba (●). Each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. In all panels the horizontal bars represent drug application. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained at times −10–0: −0.65 ± 0.06 mV/msec, $n = 3$ (*A*, ●), −0.57 ± 0.03 mV/msec, $n = 3$ (*A*, ○), −0.52 ± 0.02 mV/msec, $n = 2$ (*Ba*, ○) and −0.50 ± 0.10 mV/msec, $n = 3$ (*Ba*, ●)

experiments where the action of BDNF in the presence of ITU was tested, the neurotrophin was applied when the full effect of ITU was achieved, and the slope values of the fEPSPs recorded under these conditions were taken as 100%. As shown in Figure 5*B*, perfusion of BDNF (20 ng/ml) under these conditions resulted in a significant increase in fEPSP slope (44 ± 8.7% increase; $n = 3$; $p < 0.05$), that did not occur when A_{2A} receptors were blocked with ZM 241385 (50 nM) before ITU application ($n = 2$) (Fig. 5*B*). When ZM 241385 (50 nM) was added only 50 min after ITU, BDNF (20 ng/ml) caused the expected enhancement (51%) of synaptic transmission (one experiment). This may suggest that A_{2A} receptors are required to trigger the action of BDNF but they do not need to remain activated during the BDNF action.

The activation of the cAMP–PKA transducing system is a critical step in the excitatory action of BDNF

Because ITU and the pulse of high K^+ mimicked the action of the adenosine A_{2A} receptor agonist CGS 21680 in what respects the ability to trigger BDNF actions, and activation of A_{2A} receptors stimulates the formation of cAMP–PKA transducing system (Fredholm et al., 2001), we evaluated if a selective PKA inhibitor H-89 (Chijiwa et al., 1990) could modify the action of BDNF. H-89 (1 μM) was added for at least 30 min before K^+ pulse application (Fig. 6*A*), CGS 21680 perfusion (Fig. 6*B*), or ITU administration (Fig. 6*C*) and remained in the bath up to the end of the experiments. In all cases, H-89 abolished ($p < 0.05$) the excitatory action of BDNF on fEPSPs ($n = 3$ for each experimental condition), but by itself H-89 was virtually devoid of effect on fEPSPs (Fig. 6*D*).

To further evaluate the involvement of cAMP-dependent PKA on the synaptic action of BDNF, we tested whether a membrane-permeable cAMP analog, dibutyryl cAMP (dbcAMP) (Henion et al., 1967), influences BDNF action. By itself dbcAMP (0.5 mM) was virtually devoid of effect on fEPSP slope (Fig. 7*A*), and it was added to the slices at least 30 min before BDNF application. In the presence of dbcAMP (0.5 mM), BDNF caused a significant increase (49 ± 13.6%; $n = 4$; $p < 0.05$) in fEPSP slope (Fig. 7*B*), an effect fully blocked by H-89 (1 μM; $n = 2$; $p < 0.05$) (Fig. 7*B*). In slices predepolarized by a pulse of high-K^+ (10 mM), dbcAMP caused only a small increase on fEPSP slope (17 ± 1.4%; $n = 2$; $p < 0.05$ as compared with dbcAMP alone), suggesting that cAMP-mediated actions are slightly influenced by predepolarization.

To investigate whether the excitatory action of BDNF depends on the increase in postsynaptic levels of cAMP, postsynaptic CA1 pyramidal neurons were loaded with cAMP (0.5 mM) added to the filling solution of the patch pipette. After membrane rupture, EPSCs amplitudes gradually increased, probably because of cAMP-induced alterations in the metabolic state of the cell. In those cells that stabilized after the initial increase, bath application of BDNF (20 ng/ml) did not cause consistent changes in EPSC amplitudes (Fig. 7*C*). Thus, an increase of postsynaptic cAMP levels seems not sufficient to trigger the excitatory action of BDNF.

Discussion

The present results show that the excitatory action of BDNF on hippocampal synaptic transmission is facilitated by predepolarization and that this effect is dependent on adenosine A_{2A} receptor activation. These actions were revealed by using a BDNF concentration that on its own did not significantly modify the slope of fEPSPs and was mediated by a receptor of the tyrosine kinase receptor family, because pretreatment with the Trk receptor inhibitor K252a (Klein et al., 1990), prevented the excitatory effect of this neurotrophin on fEPSPs. BDNF possesses a greater affinity for TrkB receptors than for TrkA or TrkC receptors (Lewin and Barde, 1996) and, therefore, the observed actions of BDNF are

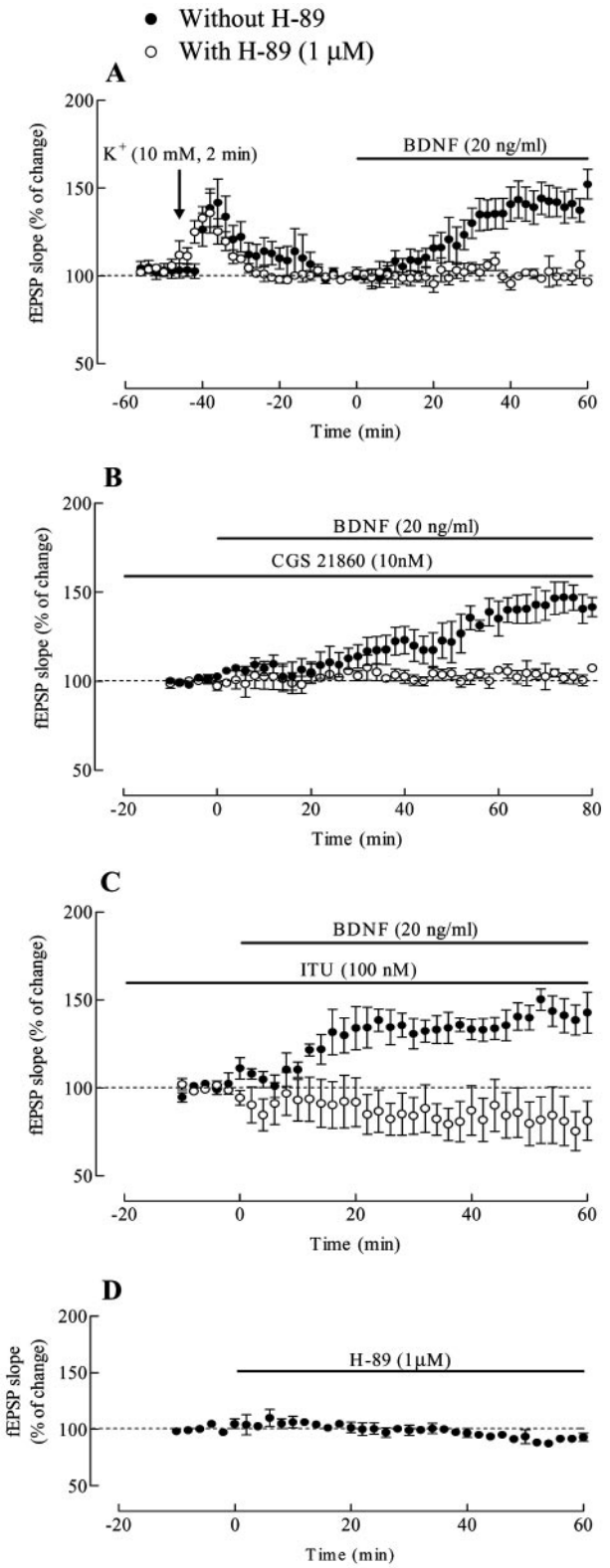

Figure 6. The activation of the cAMP–PKA transducing system is a critical step for the excitatory action of BDNF. Averaged time course of changes in fEPSP slope induced by BDNF in the absence (●) and in the presence (○) of the PKA inhibitor H-89 (1 μM) under the following experimental conditions: (*A*) after a pre-depolarizing pulse of high-K^+ (10 mM, 2 min); (*B*) in the presence of CGS 21860 (10 nM); (*C*) in the presence of 5-iodotubercidin (ITU; 100 nM). H-89 (1 μM) was added to the slices 30 min before the application of the K^+ pulse (*A*), CGS 21680 (*B*), or ITU (*C*) and alone was virtually devoid of effect on fEPSP slope, as shown in *D*. In all panels the horizontal bars represent drug application, and the arrow in *A* represents the beginning of the 2 min high-K^+ application. All values are mean ± SEM; 100% (averaged fEPSP slopes obtained at times −10–0): −0.57 ± 0.03 mV/msec, $n = 9$ (*A*, ●), −0.48 ± 0.23 mV/msec, $n = 3$ (*A*, ○), −0.61 ± 0.01 mV/msec, $n = 6$ (*B*, ●), −0.47 ± 0.06 mV/msec, $n = 3$ (*B*, ○), −0.50 ± 0.10 mV/msec, $n = 3$ (*C*, ●), −0.43 ± 0.17 mV/msec, $n = 3$ (*C*, ○), −0.59 ± 0.08 mV/msec, $n = 3$ (*D*). Note that the PKA inhibition prevented the action of BDNF in all experimental conditions.

probably caused by activation of TrkB receptors, which are present in the hippocampus (Klein et al., 1990; Lewin and Barde, 1996).

The findings that after the brief K^+-depolarizing pulse, BDNF could facilitate synaptic transmission to pyramidal neurons under voltage-clamp conditions and that BDNF was unable to facilitate synaptic transmission to neurons that had been transiently depolarized by changing the holding potential indicate that presynaptic, rather than postsynaptic, depolarization is required to gate the BDNF action. Also in accordance with the need of presynaptic depolarization to trigger BDNF excitatory action is the finding that theta burst stimulation of the Schaffer collateral afferents paired with BDNF facilitated synaptic transmission.

In accordance with our results, are the results of Boulanger and Poo (1999a) at developing neuromuscular synapses showing that presynaptic depolarization is a critical factor in causing the synaptic action of neurotrophins. This could result from the following mechanisms: (1) the prepulse of K^+ triggers secretion of endogenous neurotrophin that could act synergistically with applied BDNF to potentiate synapses; (2) potassium treatment could induce the expression of more TrkB receptors, and (3) treatment with high-K^+ induces release of adenosine (Pazzagli et al., 1993), which could gate the excitatory action of BDNF on synaptic transmission. Our results with the adenosine A_{2A} selective agonist, CGS 21680 and with ITU, a selective inhibitor of adenosine kinase that enhances the extracellular amount of adenosine (Pak et al., 1994), support this last hypothesis. Thus, in the presence of CGS 21680, BDNF increased the slope of fEPSP, even in slices that had not been predepolarized by the high-K^+ pulse. Moreover the adenosine A_{2A} receptor antagonist ZM 241385 prevented the excitatory effects of BDNF in conditions of predepolarization, as well as in the presence of CGS 21680. Finally, ITU was also able to unmask the BDNF excitatory action, as did CGS 21860 and the brief K^+ pulse, an action also prevented by the A_{2A} antagonist ZM 241385.

There are two sources of extracellular adenosine: release of adenosine by facilitated diffusion, through membrane adenosine transporters that are equilibrative and bidirectional (Gu et al., 1995), and extracellular conversion of released adenine nucleotides into adenosine through a series of ectoenzymes, the last one in the cascade and the rate-limiting step for adenosine formation being ecto-5′-nucleotidase (Zimmermann and Braun, 1999). ATP is co-stored with most neurotransmitters, and therefore neuronal activity causes the release of ATP and extracellular formation of adenosine in most brain areas, including the hippocampus (Wieraszko et al., 1989; Cunha et al., 1996). Depolarization by K^+ predominantly induces the release of adenosine as such (Latini and Pedata, 2001). The intracellular adenosine levels are kept low mainly because of the activity of adenosine kinase (Arch and Newsholme, 1978) and when this enzyme is inhibited (e.g., by ITU) there is a marked increase in the release of adenosine (Pak et al., 1994). One may question why K^+-induced adenosine release did not cause an A_1-receptor mediated decrease in synaptic transmission, as ITU did. This most probably results from the time course of A_1-receptor mediated actions (Sebastião

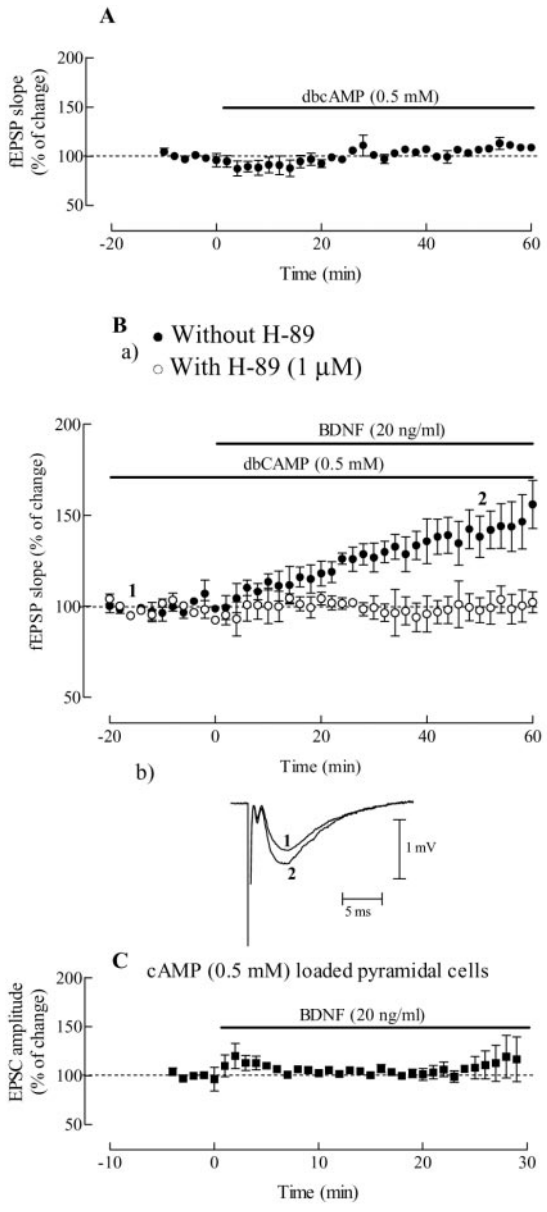

Figure 7. Presynaptic cAMP mimics the effect of high-K^+, ITU, or CGS 21680. *A* shows the averaged time course of changes in fEPSP slope induced by application of dbcAMP (0.5 mM), and *Ba* shows the averaged time course of changes in fEPSP slope induced by BDNF (20 ng/ml) in the presence of dbcAMP (0.5 mM), either in the absence (●) and or the presence (○) of the PKA inhibitor H-89 (1 μM). Slices were superfused with dbcAMP 30 min before BDNF application. *Bb* shows traces obtained in a representative experiment in *Ba* (●); each trace is the average of eight consecutive responses obtained immediately before (1) and during (2) BDNF application, and is composed of the stimulus artifact, followed by the presynaptic volley and the fEPSP. *C* illustrates the averaged time course of changes induced by BDNF (20 ng/ml) on the amplitude of EPSCs recorded from postsynaptic pyramidal neurons loaded with cAMP, which was added (0.5 mM) to the whole-cell recording pipette. In all panels the horizontal bar represents the application of the different drugs. All values are mean ± SEM; 100% in *A* and *B* [averaged fEPSP slopes obtained at times −10–0: −0.70 ± 0.2, $n = 2$ (*A*), −0.56 ± 1.60 mV/msec, $n = 4$ (*Ba*, ○), and −0.64 ± 0.04 mV/msec, $n = 2$ (*Ba*, ●)]; 100% values in *C* (EPSC amplitudes recorded at times −5–0) ranged from −500 to −1400 pA; $n = 2$. Note that BDNF enhanced synaptic transmission in the presence of the cell-permeant cAMP analog, dbcAMP, but when cAMP was present only postsynaptically (*C*) it did not trigger the action of BDNF.

et al., 1990; Lupica et al., 1992): any fast inhibitory actions, because of adenosine released during the 2 min application of K^+, should be masked below the transient excitation induced by depolarization. In contrast, activation of A_{2A} receptors for a similar period of time might lead to long lasting enhancement in intracellular cAMP caused by adenylate cyclase activation (Fredholm et al., 2001), which could gate BDNF actions (Boulanger and Poo, 1999b). In line with this possibility are our observations that the PKA inhibitor, H-89, prevented the excitatory action of BDNF in the presence of K^+ pulse, CGS 21680, or ITU. Moreover in the presence of the cAMP analog dbcAMP, BDNF enhanced synaptic transmission, an effect blocked by H-89. Presynaptic, rather than postsynaptic enhancement of cAMP might be required to trigger the action of BDNF because the neurotrophin was unable to enhance synaptic currents in cAMP loaded pyramidal cells.

It is known that cAMP can induce a variety of cellular processes in different systems, including expression of mRNA for Trk receptors and neurotrophins in primary astroglial cultures (Condorelli et al., 1994) and recruitment of TrkB receptors to the plasma membrane of CNS neurons (Meyer-Franke et al., 1998). Cytosolic cAMP can be positively modulated by depolarization and synaptic activity (Ferrendelli et al., 1980), and activation of cAMP signaling has been shown to potentiate facilitatory actions of BDNF at the developing neuromuscular junction (Boulanger and Poo, 1999b). A cAMP—PKA—CREB-dependent pathway is also shown to be involved in the ability of adenosine A_{2A} receptor agonists to potentiate nerve growth factor-induced neurite outgrowth in cultured PC12 cells (Chen et al., 2002). The results now described show that the interactions between neurotrophins and adenosine A_{2A} receptors can occur in CNS neurons that were not in culture, therefore not at an active developing stage. The finding that blockade of adenosine A_{2A} receptors prevents the action of BDNF after a K^+ predepolarizing pulse, suggests that the predepolarization pulse *per se* is not an essential requisite to unmask BDNF excitatory effects, eventually by a K^+-induced increase in cAMP levels (Ferrendelli et al., 1980). In contrast, activation of adenosine A_{2A} receptors either by released adenosine or by a selective agonist (CGS 21680) appears to be an essential step to trigger BDNF excitatory action on synaptic transmission.

Clear excitatory actions of BDNF on hippocampal synaptic transmission have been observed by some authors (Kang and Schuman, 1995, 1996), whereas others reported minimal or no effects of this neurotrophin in the same brain area (Figurov et al., 1996; Gottschalk et al., 1998). Whether this discrepancy results from different levels of endogenous activation of adenosine A_{2A} receptors, awaits further investigation.

Neurotrophins have been suggested to have an important role in protecting mature neurons from neuronal atrophy in the degenerating human brain. A decrease in BDNF levels might be involved in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease (Connor and Dragunow, 1998), or diabetic neuropathies (Nitta et al., 2002), making the use of the naturally occurring neurotrophic factors very promising for treatment of these disorders. However, until now the pharmacological administration of *in vivo* BDNF has not been easy. One of the reasons is that these molecules are unable to cross the blood–brain barrier, making invasive application strategies like intracerebroventricular infusion necessary. Intravenous administration of BDNF has been attempted, but it involves a complex molecular reformulation of the neurotrophin (Wu and Pardridge, 1999). The results now described open a new prospective to potentiate BDNF actions on CNS neurons, i.e., by co-activation of a specific type of adenosine receptors, the A_{2A} receptors. This possibility expands the pathophysiological implications of adenosine receptor functioning in the brain (Ribeiro et al., 2003) and points toward new strategies to interfere with neu-

rotrophic factors in the therapeutics of some neurodegenerative disease.

In conclusion, our results suggest a way to enhance the excitatory action of BDNF on synaptic transmission via activation of adenosine A_{2A} receptors. Through activation of these receptors it would be possible to overcome some difficulties with the use and delivery of neurotrophic factors when neuroprotection by these agents is required.

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