BDNF Acutely Modulates Synaptic Transmission and Calcium Signalling in Developing Cortical Neurons

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Key Words

BDNF • Calcium • Cultured cortical neurons • Synaptic plasticity • Voltage sensitive Ca^{2+} channels

intracellular calcium stores and influx of extracellular Ca^{2+} mainly through voltage-dependent Ca^{2+} channels in cultured cortical neurons.

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Abstract

Brain-derived neurotrophic factor (BDNF), like other neurotrophins, has long-term effects on neuronal survival and differentiation; furthermore, BDNF has been reported to exert an acute potentiation of synaptic activity and are critically involved in long-term potentiation (LTP). We found that BDNF rapidly induced potentiation of synaptic activity and an increase in the intracellular Ca^{2+} concentration in cultured cortical neurons. Within minutes of BDNF application to cultured cortical neurons, spontaneous firing rate was dramatically increased as was the frequency and amplitude of excitatory spontaneous postsynaptic currents (EPSCs). Fura-2 recordings showed that BDNF acutely elicited an increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$). This effect was partially dependent on extracellular Ca^{2+} . In calcium-free perfusion medium a substantial calcium signal remained which disappeared after loading of cortical neurons with 5 μM U-73122. BDNF-induced Ca^{2+} transients were completely blocked by K252a and partially blocked by Cd^{2+} . The results demonstrate that BDNF can enhance synaptic transmission and induce directly a rise in $[\text{Ca}^{2+}]_i$ that require two routes: the release of Ca^{2+} from

Introduction

Neurotrophins are a family of neurotrophic proteins that includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 [1]. The neurotrophins mediate their actions by two types of receptors; that is, Trk receptor tyrosine kinases and the pan-neurotrophin receptor p75NTR. Each type of Trk is activated by one or several neurotrophins: TrkA by NGF, TrkB by BDNF or neurotrophin-4/5, and TrkC by neurotrophin-3. This specific binding leads to the receptor dimerization, activation, and recruitment of various adapter proteins to activate a wide range of signaling pathways. The role of neurotrophins as regulatory factors that mediate differentiation and survival of neurons in the peripheral and central nervous system is well established [2, 3]. Besides having long-term trophic functions, neurotrophins play pivotal roles in acute and long-term changes in synaptic plasticity [4, 5].

In particular, BDNF acutely modulates the efficacy of basal synaptic transmission at developing neuromuscular junctions in cell cultures [6] and at central excitatory synapses in hippocampal and cortical cultures [7-9], acute slices [10, 11], and in vivo [12]. However, different systems show diverse responsiveness to BDNF and some discrepancies have yet to be clarified. In addition, brain-derived neurotrophic factor (BDNF) plays an important role in activity-induced long-term potentiation (LTP) in the hippocampus, and might be necessary for the conversion of early-phase LTP to late-phase, protein synthesis-dependent, LTP [13].

In the present study we have investigated the effects of BDNF on synaptic transmission and calcium signaling in culture cortical neurons. We find that BDNF rapidly enhanced the strength of synaptic connections in cortical neurons. Both the amplitude and the frequency of excitatory postsynaptic currents (EPSCs) were increased within 3-5 min of BDNF application. Our data also show that BDNF acutely elicited an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), which is dependent on both the influx of extracellular calcium and calcium mobilization from intracellular calcium stores.

Materials and Methods

Cell culture

Primary cultures of cortical neurons were prepared from embryonic rats (E18) by a modification of the method previously described [14]. In brief, cortical tissues from 18 d fetal rats were dissected and treated with 0.25% trypsin and 0.25 mg/ml DNase (Sigma, St. Louis, MO) in Ca^{2+} - Mg^{2+} -free HBSS at 37°C for 15 min; they were then dissociated by trituration with a glass Pasteur pipette and plated at a density of 4 or $5 \times 10^5/cm^2$ in 35 mm culture dishes with glass coverslips. The glass coverslips in each dish was pretreated with poly-D-lysine (Sigma) for 2 hr (500 $\mu g/ml$ in borate buffer), washed three times, and air-dried before cell plating. The plating medium consisted of Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA), 500 μM Glutamax (Invitrogen), 10% fetal bovine serum and 10% horse serum (Hyclone, Logan, UT). On the second day after plating, the culture medium was replaced by DMEM containing 500 μM Glutamax (Invitrogen) and 5% horse serum. Cells were maintained in a CO_2 incubator at 37°C, and the culture medium was replaced with DMEM containing 500 μM Glutamax and 5% horse serum every 3d.

Electrophysiological recordings

Whole-cell current-clamp and voltage-clamp recordings were obtained from pyramidal-type cells after 10-14 days in vitro with standard techniques [15]. The external bath solution contained 145 mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$,

10mM glucose, and 10mM HEPES, pH 7.2. The pipette solution contained 145 mM KCl, 0.1mM $CaCl_2$, 11 mM EGTA, 1mM $MgCl_2$, 10mM HEPES, and 2mM ATP, pH 7.2. Signals were recorded with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) by using patch pipettes (resistance 4–5 M Ω) filled with the pipette solution. For voltage-clamp experiments, cells were held at membrane potential of -60mV. The series resistance was 8–15 M Ω . All experiments were performed at room temperature.

Intracellular calcium measurement

Intracellular calcium ($[Ca^{2+}]_i$) levels of single neuron were measured by using fura-2 AM and ratiometric imaging techniques [16]. Cells were loaded for 40 min at 37°C with Fura-2AM (2 μM , Molecular Probes, Eugene, OR) dissolved in dimethyl sulfoxide/10% pluronic F-127 (Molecular Probes), rinsed, and incubated in fresh culture medium for 10 min before the measurement. During $[Ca^{2+}]_i$ measurement, cells were kept in modified Hanks' buffer. The fluorescence setup was as follows: the fluorescence microscopy is built around an Axiovert 100 (Zeiss, Germany); excitation light 345nm/380nm is provided by a monochromatic system (TILL Photonics, Germany). The fluorescence signals are collected by a photomultiplier and converted to voltage values, then digitized and sent to the computer. X-chart (HEKA, Germany) is used to record the signal. Intracellular free Ca^{2+} concentration was measured according to the following equation: $[Ca^{2+}]_i = K_{eff} (R - R_{min}) / (R_{max} - R)$. K_{eff} is effective binding constant. R is a ratio of fluorescence at two wavelengths (at excitation wavelengths of 340 and 380 nm). R_{min} is the limiting value that the ratio can have at zero $[Ca^{2+}]_i$. R_{max} is the limiting value that the ratio can have at saturating $[Ca^{2+}]_i$ (10mM). In our experiment, R_{min} was 0.1, R_{max} was 3.6, and $K_{eff} = 1918$ nM, as determined by standard calibrations.

BDNF was extracellularly applied by puffer pipettes. The puffer pipette tip (5 μm in diameter) was set 30 μm away from the cell.

Drugs

Brain-Derived Neurotrophic Factor (BDNF) was purchased from Sigma. K-252a and U-73122 were obtained from Calbiochem. All other chemicals were from Sigma. The duration for BDNF application was 30s. U-73122 (5 μM) was added 30 min prior to the application of BDNF. K252a (200 nM) was added 20 min prior to the intracellular calcium measurement.

Results

BDNF increases spontaneous activity in dissociated cultures of cortical neurons

To investigate the role of BDNF in regulating the overall excitability of dissociated cultures of cortical neurons, spontaneous action potentials in neurons with pyramidal morphology were recorded using whole-cell current-clamp configuration. Bath application of BDNF at 100ng/ml increased the average spontaneous action

Fig. 1. Potentiation of neuronal activity by BDNF. (A) Voltage traces recorded from a cortical neuron under a current-clamped mode. Treatment of BDNF(100 ng/ml) markedly increased the spike frequency. Top trace: a representative voltage trace before the application of BDNF was shown; middle trace: a voltage trace after the bath application of BDNF was shown; bottom trace: a recorded voltage trace after washout of BDNF for 6 min was shown. (B), Mean group data for the effect of BDNF on firing rate (n=10 cells; **p<0.01).

potential firing rates of neurons within 3~5 min of application (Fig. 1A). This effect appeared 3-5 min after the addition of BDNF and gradually recover to baseline levels within 3-8 min after BDNF washout. On average, firing rates increased >8-fold during BDNF exposure (Fig. 1B)[the mean control firing rate = 1.75 ± 1.2 spikes

Fig. 2. Potentiation of synaptic transmission by BDNF. (A) Shown is an example of recordings. The effect of BDNF (100 ng/ml) on the EPSCs appeared 3 min after application. The increased frequency and the increased magnitude recovered to baseline ~5 min after washout of BDNF. (B) Mean group data for the effect of BDNF on EPSCs frequency (n=9 cells; **p<0.01). (C) Mean group data for the effect of BDNF on EPSCs magnitude (n=9 cells; p<0.01).

per 10 sec; the mean firing rate during BDNF application = 14.82 ± 4.2 (mean $\pm$ SD; n=10; p<0.01). This is consistent with the finding of Levine [8].

BDNF increases the frequency and the amplitude of EPSCs

The cortical cultures contain networks of interacting neurons. Whole-cell recordings ($V_h = -60$ mV) in one cell of the network revealed spontaneous synaptic activity in the vast majority of neurons after 1 weeks in culture (Fig. 2A). These currents represent action potential-dependent spontaneous EPSCs mediated by non-NMDA receptors, because they were abolished by 20 μ M CNQX, a non-NMDA receptor antagonist (data not shown). Bath application of BDNF at 100 ng/ml increased the frequency of the spontaneous EPSCs. This effect appeared 3-5 min after the addition of BDNF; gradual recovery to baseline levels occurred within 3-8 min after BDNF washout (Fig. 2A). On average, the rates of spontaneous EPSCs increased to $317 \pm 5\%$ of control levels [the mean control EPSCs rate = 10.25 ± 2.7 number

Fig. 3. BDNF induced an increase in intracellular Ca^{2+} . (A) A cortical neuron responded to BDNF with an increase in $[\text{Ca}^{2+}]_i$. (a) active neuron which have spontaneous Ca^{2+} spikes (b)silence cortical neuron. (c) continuous application of BDNF is necessary for the maintenance of a sustained elevation of intracellular Ca^{2+} . (B) Under the existences of K252a, BDNF (100 ng/ml) failed to induce Ca^{2+} transients. The arrow in each panel indicates the beginning of BDNF application. The bar in each panel indicates the period of BDNF application.

Fig. 4. (A) In calcium-free perfusion medium a substantial calcium signal remained (b), which disappeared after loading of cortical neurons with U-73122(c). (B) Under the existences of Cd^{2+} , BDNF still induced Ca^{2+} increase. However, BDNF-induced Ca^{2+} transients were partially blocked by Cd^{2+} (b). The arrow in each panel indicates the beginning of BDNF application. The bar in each panel indicates the period of blockers application.

Fig. 5. (A) TTX had no effect on BDNF-induced Ca^{2+} transients. (B) BDNF-evoked Ca^{2+} signals were insensitive to a cocktail of glutamate receptor blockers CNQX and APV. (C) BDNF rapidly evoked membrane subthreshold depolarization (a) and almost synchronously induced a transient calcium signal (b) in cortical neurons. The arrow in each panel indicates the beginning of BDNF application. The bar in each panel indicates the period of BDNF application.

per 10 sec; the mean EPSCs rate during BDNF application = 31.59 ± 6.5 (mean $\pm$ SD; $n=9$; $p < 0.01$) (Fig. 2B). In addition, the amplitudes of the spontaneous EPSCs were increased to $192 \pm 10\%$ of control levels by BDNF application (Fig. 2C).

BDNF results both in influx of extracellular calcium and calcium mobilization from intracellular stores

This experiment was designed to characterize BDNF-induced calcium signaling in single cortical neurons in culture. After 30 sec of BDNF application (100 ng/ml) the neurons showed a rapid rise in the concentration of $[\text{Ca}^{2+}]_i$, both in dendritic processes and cell bodies of cortical neurons ($n=10$, Fig. 3A); this elevation was reversed with the washout of BDNF and could be elicited repetitively (Fig. 3A) and continuous application of BDNF is necessary for the maintenance of a sustained elevation of intracellular Ca^{2+} (Fig. 3A(c)). At the same time, we found BDNF can elicit calcium transients both in silence cortical neuron and active neuron which have spontaneous Ca^{2+} spikes (Fig. 3A(a),(b)). In the presence of 200 nM K252a, a membrane-permeable inhibitor of Trk receptor tyrosine kinase, BDNF (100 ng/ml) failed to elevate intracellular Ca^{2+} concentration in cortex neurons ($n=6$; Fig. 3B). Thus, a K252a sensitive pathway mediates the

induction of the intracellular Ca^{2+} elevation, probably involving the Trk B receptor tyrosine kinase activity.

In calcium-free perfusion medium (0mM calcium, 5mM EGTA) a substantial calcium signal remained (Ca^{2+} transients were reduced by $58\% \pm 8\%$, $n=6$, Fig. 4A(b)), which disappeared after loading of cortical neurons with 5 μM U-73122 (a membrane-permeable specific PLC- γ inhibitor) (Fig. 4A(c)), indicating that the signal partially was caused by calcium mobilization from intracellular stores through the PLC- γ /IP3 pathway. Under the existences of Cd^{2+} (voltage-dependent Ca^{2+} channel blocker, 100 μM), BDNF still induced Ca^{2+} increase (Cd^{2+} reduced the calcium transients by $38\% \pm 10\%$, $n=6$, Fig. 4B). BDNF-evoked Ca^{2+} signals were altered neither by tetrodotoxin ($404 \pm 102 \text{ nM}$ without TTX and $402 \pm 99 \text{ nM}$ with TTX; $n=5$), nor by a cocktail of glutamate receptor blockers CNQX and APV ($393 \pm 84 \text{ nM}$ without CNQX/APV and $391 \pm 83 \text{ nM}$ with CNQX/APV; $n=5$) (Fig. 5A,B). We also found BDNF could rapidly evoke membrane subthreshold depolarization and almost synchronously induce a transient calcium signal in cortical neurons (Fig. 5C). All the above results indicate BDNF-evoked Ca^{2+} signals depend on calcium mobilization from intracellular stores and the influx of extracellular calcium may be mainly controlled by voltage-dependent Ca^{2+} channels.

Discussion

Neurotrophins play a fundamental role in the survival and differentiation of many types of neurons. In addition to developmental regulation, neurotrophins are also important factors in synaptic plasticity and memory formation. Moreover, BDNF is essential to synaptic transmissions, activity-dependent synaptic plasticity and long-term memory [4, 5]. Besides potentiate excitatory synaptic transmission [7, 8], application of BDNF to neurons also can activate a TRPC3-dependent cation current [17], cause membrane depolarization within a few milliseconds through TrkB activity-dependent activation of a novel form of membrane sodium channels [18], and induce Ca^{2+} mobilization [19-21]. Many studies subsequent to that of Lohof et al [6] have shown that BDNF and other neurotrophins can modulate synaptic transmission in cultured neurons [22-24], hippocampal slices [25-27], visual cortex slices [11, 28], and hippocampal neurons in vivo [12]. However, in these studies, there are some inconsistency and varied results in the reported acute effects on apparently similar systems, which may be due to differences in the preparations and experimental procedures. Levine et al [8] reported that BDNF enhanced excitatory synaptic transmission in cultured hippocampal neurons. In rat hippocampal slices, Tanaka et al [29] described the inhibition of GABA_A synaptic responses by BDNF. Frerking et al also reported BDNF modulated inhibitory, but not excitatory, transmission in the CA1 region of the hippocampus [27]. BDNF acutely inhibited AMPA-mediated currents in developing sensory relay neurons was reported by Balkowiec et al [30]. As far as BDNF-induced Ca^{2+} mobilization, there are also some varied results have been reported. Li et al [9] reported BDNF induced intracellular Ca^{2+} elevation depended on extracellular Ca^{2+} in hippocampal neurons. Numakawa et al [21] reported BDNF-induced Ca^{2+} elevation was mediated by IP3 receptor rather than by an influx of extracellular Ca^{2+} . Mizoguchi et al [20] found a brief BDNF could cause sustained intracellular Ca^{2+} elevation in rat visual cortex neurons, which was dependent on intracellular Ca^{2+} stores.

In present study we investigated the effects of BDNF on synaptic transmission and calcium signaling of cortical neuron. We found that BDNF rapidly induced potentiation of spontaneous neuron activity of cortical network. Within minutes of BDNF application to cultured cortical neurons, spontaneous firing rate was dramatically

increased as was the frequency and amplitude of excitatory spontaneous postsynaptic currents (EPSCs), which is consistent with previous reports [8, 28]. In agreement with earlier observations [19-21], we found that BDNF could also rapidly induce an increase in the intracellular Ca^{2+} concentration in cultured cortical neurons. In calcium-free perfusion medium, a substantial calcium signal remained, which disappeared after loading of cortical neurons with 5 μM U-73122, it suggested that the signal partially was caused by calcium mobilization from intracellular stores through the PLC- γ /IP3 pathway. In addition, BDNF-induced Ca^{2+} transients were partially blocked by Cd^{2+} . All data indicate that BDNF-evoked Ca^{2+} signals result from the influx of extracellular calcium might mainly through voltage-sensitive Ca^{2+} channels and the calcium mobilization from intracellular stores.

In neurons of CNS, the binding of BDNF to TrkB can activate the non-selective cation channel TRPC3 through PLC- γ /IP3 pathway [17]. Kafitz et al reported another, much faster and distinctly different excitatory action of neurotrophins. In various types of CNS neurons, the study found that low nanomolar levels of BDNF evoke neuronal excitation by activating a Na current (I_{Na}). This BDNF-evoked I_{Na} underlying neuronal depolarization was activated within milliseconds and, as a function of the BDNF dose and the time of application, resulted in trains of action potentials [18]. One report identified dendritic spines as highly sensitive sites for the BDNF-evoked excitation, and demonstrated that in hippocampal dentate granule cells induction of BDNF-mediated LTP occurred post-synaptically [31]. Recently, one report demonstrates that the ligand BDNF, bound to the receptor tyrosine kinase TrkB, can evoke membrane depolarization through rapid activation of the sodium channel NaV1.9 [32]. In our experiments, we found BDNF could rapidly evoke membrane subthreshold depolarization and almost synchronously induce a transient calcium signal in cortical neurons. We also found that BDNF-evoked Ca^{2+} signals in cortical neuron were altered by neither tetrodotoxin, nor by a cocktail of glutamate receptor blockers CNQX and APV. Therefore, it suggests that both of BDNF-evoked membrane depolarization and calcium mobilization from intracellular stores through PLC- γ /IP3 pathway may be responsible for BDNF-evoked Ca^{2+} signals. The mechanisms of BDNF-evoked membrane depolarization in cultured cortical neurons will be investigated in our future experiments.

Previous studies have investigated whether BDNF-induced synaptic potentiation occurs pre- or post-

synaptically. Some studies indicated that the BDNF modification of synaptic transmission occurred presynaptically [6, 7, 9, 33]. On the other hand, some studies indicated postsynaptic actions of BDNF. Levine et al. demonstrated BDNF modulated hippocampal synaptic transmission by increasing NMDA receptor activity [23]. BDNF, within 5 min of exposure, elicited a dose-dependent increase in phosphorylation of the N-methyl-D-aspartate receptor subunit [34, 35]. Recently, Wu et al. reported Brain-derived neurotrophic factor acutely enhanced tyrosine phosphorylation of the AMPA receptor subunit GluR1 via NMDA receptor-dependent mechanisms [36]. In our experiment, we found some interesting thing about the onset/time scale of the BDNF effect: a 30 second BDNF application could induce a transient calcium signal, while it took 3 to 5 minutes for BDNF to induce synaptic potentiation. We also found the time needed for BDNF-induced synaptic potentiation was consistent with the time for BDNF to enhance the phosphorylation of NMDA receptor subunit 1 and AMPA receptor subunit GluR1 [34–36]. In addition, Kovalchuk et al. demonstrated BDNF pulses failed to induce any

transient calcium signals when applied to the axon [31]. Thus, enhancement of the NMDA and the AMPA receptor activity through phosphorylation by BDNF was a better explanation for BDNF-induced potentiation of the frequency and the amplitude of EPSCs than the presumption that BDNF-induced transient calcium signals increased the release of presynaptic vesicles. Namely, our results support the postsynaptic mechanism. However, BDNF-induced transient calcium signals may play an important role in signal cascades of the phosphorylation of NMDA receptor and AMPA receptor by BDNF. Future experiments that block BDNF signal pathway selectively in pre- or postsynaptic neurons will address this question directly.

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