Possible involvement of BDNF release in long-lasting synapse formation induced by repetitive PKA activation

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Abstract

For the analysis of the cellular mechanism underlying long-term synaptic plasticity, a model system that allows long-lasting pursuit is required. Previously we reported that, in hippocampal neurons under dissociated cell culture conditions, repeated (but not a single) transient activation of protein kinase A (PKA) led to an increase in the number of synapses that lasted ≥3 weeks, and hence we proposed that this phenomenon should serve as an appropriate model system. Here we report that repeated pulsatile application of brain-derived neurotrophic factor (BDNF) leads to persistent synapse formation equivalent to that after the repeated transient activation of PKA. A BDNF-scavenging substance applied concomitantly with PKA activation abolished the synapse formation. The release of BDNF upon PKA activation was confirmed by phosphorylation of TrkB. These results indicate that the release of BDNF is involved in the putative signaling cascade connecting PKA activation and synapse formation.

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Synaptic plasticity, a cellular substrate of brain memory, is based on increased transmission efficiency in existing synapses in the short term and on the formation of new synapses in the long term [2,6]. However, the cellular mechanisms leading to synapse formation have not been well understood due primarily to the lack of a good model system for analysis. For a long-term investigation of cellular processes, a stable neuronal culture should be useful. Previously, we found that in organotypic brain slice cultures of the rat hippocampus, that three transient activations of cyclic AMP-dependent protein kinase (PKA) at proper intervals (ranging from 3 to 24 h), led to an enhancement in synaptic transmission strength that developed slowly and lasted ≥3 weeks [23]. This enhancement was accompanied by an increase in the number of synapses. Recently we made the additional finding that a similar synapse formation was induced after three activations of PKA at similar intervals in dissociated neuronal cultures of the rat hippocampus [25]. Dissociated cell cultures are advantageous for live examination of ongoing cellular processes [15]. Thus, we proposed that these synaptogenic phenomena in slice and dissociated cell cultures should serve as good model systems for the analysis of long-term synaptic plasticity.

Among many possible outputs downstream of PKA activation [12], the release of brain-derived neurotrophic factor (BDNF) is included [16]. BDNF, one of the most intensively-studied neurotrophic factors [3], is known to induce long-term potentiation (LTP) in acutely prepared hippocampal slices [11], although LTP is a form of plasticity different from the present phenomenon. Tonic exposure to BDNF is known to induce synapse formation [1,18]. Taken together, it is reasonable to postulate that the repetition of transient releases of BDNF mediates the formation of new synapses after the repetition of transient activation of PKA.

We examined whether three brief applications of BDNF would induce synapse formation equivalent to that of after three transient activations of PKA. We also examined the effect of a BDNF-scavenging substance (a chimeric protein between high-affinity BDNF receptor TrkB and immunoglobulin Fc segment [13]) applied concomitantly with PKA activation, as it should block the development of synaptogenesis if BDNF release is responsible for PKA-dependent synaptogenesis. Both of these expectations were confirmed.
Following the protocols of our previous report [25], the hippocampi were dissected out from E18–19 Wistar/ST rat embryos (Nihon SLC). Cells were dissociated by trypsinisation and plated at the density of $6.6 \times 10^3$ cm$^{-2}$ onto polyethyleneimine-coated circular glass coverslips (ø13 mm; sunk in each well of ø15 mm multi-well plates). Cultures were maintained using a Neurobasal serum-free medium (Gibco) mixed with 2% B27 supplement (Gibco) for 21 days, during which period the neurons formed stable synaptic connections [25]. Then we applied drugs (see below) once or three times at 24-h intervals. Throughout this study, the number of days after stimulation was reckoned from the day of the first application of drug(s). The drugs used were: recombinant human BDNF (100 ng/ml, 60 min, supplied from Regeneron), Sp-3′,5′-cyclic adenosine phosphorothioate (Sp-cAMPS, a membrane permeable analogue of cyclic AMP and a PKA activator, 50 µM, 15 min, supplied from Biolog) or TrkB-Fc (a BDNF scavenger, 0.4 µg/ml, applied for 24 h beginning 20 min before Sp-cAMPS application). TrkB-Fc dosage was determined from the supplier’s (R&D Systems) protocol. The cultures were washed free from the drugs by two consecutive renewals of the medium.

After a predetermined days (3, 9 or 14 days) had passed, the cultures were fixed with 4% paraformaldehyde and processed for conventional immunocytochemical staining using antibodies against synaptophysin (a presynaptic marker protein, 1/500, polyclonal, Santa Cruz) and PSD-95 (a postsynaptic marker protein for excitatory synapses, 1/100, monoclonal, Sigma). AF488-conjugated anti-mouse-IgG and AF546-conjugated anti-rabbit-IgG were used as secondary antibodies. An Olympus BX50 fluorescence microscope was used to take photographs of pyramidal neurons (identified by their large triangular somata). The sites of apposition between punctate structures stained for the two respective markers were identified as synapses (arrows in Fig. 1; since pre- and postsynaptic structures are distinct entities, they do not necessarily merge completely) and enumerated. The region of interest for enumeration was fixed on a segment of neurite between the first and second branching points, which should correspond to the secondary dendritic segment of the pyramidal neuron in vivo.

For immunoblotting of the total and phosphorylated form of TrkB, cultures prepared on a larger scale (5 × 10$^6$ cells in a ø60-mm dish) were solubilized with ice-cold lysis buffer. The supernatant was boiled for 5 min in PAGE-sampling buffer, electrophoresed on an 8% SDS–PAGE slab gel plate, and then transferred onto a polyvinylidene difluoride membrane. The membrane was treated sequentially with a rabbit antibody recognizing specifically TrkB phosphorylated at tyrosine-490 (diluted to 1/1000, Santa Cruz) and a horseradish-peroxidase-conjugated antibody to rabbit IgG (1/3000, Bio-Rad). Immunoreactivity was visualized using a chemiluminescence kit (ECL, Pierce) and quantified by photometry using a LAS1000 image analyzer (Fuji Film). For re-probing, the membrane was treated with a stripping buffer, followed by sequential treatment with a rabbit antibody recognizing all forms of TrkB (1/250, Santa Cruz) and anti-rabbit IgG antibody. Animals were treated properly according to our institutional guidelines for animal welfare.

The density of putative excitatory synapses (sites of apposition between synaptophysin-positive and PSD-95-positive punctate structures) of the culture 14 days after three brief exposures to BDNF was higher than that of the day-matched 3 × mock-stimulated culture (Fig. 1). A single exposure to BDNF produced no increase in the synapse density as monitored at 14 day after the exposure.

The quantitative comparison of the synapse densities of the cultures exposed 1 × and 3 × to BDNF are shown in Fig. 2A. The synapse density was higher in the 3 × BDNF-exposed cultures than that in the 1 × BDNF-exposed cultures at 3 day after the first exposure to BDNF (i.e. 1 day after the third exposure) and this difference was maintained for a long period (as long as ≥ 14 day).

The interpretation of the above results in connection with the synaptogenic effect of BDNF would be complicated if the number of living neurons were affected by the exposure to BDNF. In fact, chronic exposure to BDNF is known to exert a neuroprotective effect upon an ischemic insult [17] and the

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Fig. 1. Fluorescence micrographs of 3 × mock-stimulated (A) and 3 × BDNF-exposed (B) hippocampal neurons at 14 day after stimulation. Synaptophysin- and PSD-95-immunopositive punctate structures are identified by red and green fluorescence, respectively. Three right-hand images in each row are magnified views of the boxed area (i.e. region of interest, ROI) in the leftmost image. The ROI is a segment of neurite between the first and second branching points. The ROI length of 15 µm was chosen, since the segment was sometimes less than 20 µm in dissociated cell cultures. Arrows indicate the sites of apposition between synaptophysin- and PSD-95-positive puncta, which we defined as synapses. Bars indicate 10 µm.
viability of cultured cells are decreased in BDNF knock-out mice when compared with wild-type mice [10]. In addition, since the hippocampal neurons under culture can be both presynaptic and postsynaptic and hence can form synaptic connections among them [14], the apparent synaptic density could be influenced by the number of neurons in the vessel. However, the numbers of neurons were the same among the 1× BDNF-exposed, 3× BDNF-exposed and mock-stimulated cultures (5.0±0.1, 5.0±0.3 and 5.2±0.2 [×10^3/cm^2], N=6), respectively). The absence of a survival-promoting effect of BDNF might have been due to the limited time span of the exposure.

Thus, it is concluded that brief exposure to BDNF, when repeated, leads to a persistent synapse formation that is apparently equivalent to that after repeated activation of PKA. However, this does not necessarily mean that PKA activation causes BDNF release. To examine this possibility, we applied a BDNF scavenging substance (TrkB-Fc) simultaneously with activation of PKA. When TrkB-Fc was applied during/after the administration of Sp-cAMPS, the synaptogenic effect of the PKA activator was abolished (Fig. 2B). TrkB-Fc, by itself, had no effect on synapse formation.

Previously we reported that the repetition of transient activation of PKA led to a persistent synapse formation in hippocampal neurons in both organotypic slice cultures and dissociated cell cultures [19,23,25]. The results of the present study indicate that the release of BDNF is a possible candidate for the output of PKA activation leading to the synapse formation. At the same time, this is the first demonstration of a long-lasting synaptogenic effect of transient exposure to BDNF in a dissociated neuronal culture system. The functionality, however, of newly-formed synapses remains unknown. Examination using a fluorescent dye FM1-43 showed that the synapse newly formed after three exposures to Sp-cAMPS were capable of exocytosis [25]. One of the present authors reported previously in a dissociated neuronal culture of cerebral cortex that BDNF increased...
the amount of synaptic vesicle proteins and the release of glutamate triggered by depolarization [21]. Although the duration of BDNF exposure is different (3 × 60 min in the present study versus chronically 5 day in the previous study), this suggests the functionality of the synapse newly formed after three exposures to BDNF. However, there remains a possibility that the postsynaptic compartment would be silent [7].

The reason for the necessity of repetition of PKA activation is thus far unknown. It is worth mentioning here that the repetition must be three times and must be spaced at proper intervals [23,25]. This suggests the requirement of stepwise protein synthesis after each PKA activation. The necessity of repetition also in the case of BDNF application suggests that BDNF is involved in the PKA signaling pathway, but not as the final output of the third PKA activation. It is possible to postulate that PKA activation induces BDNF-mediated LTP each time [11,16] and that the repetitive induction of LTP leads to persistent synapse formation. Although this is a plausible postulation, it is not so far established that PKA activation or BDNF application can cause LTP in dissociated cell cultures. It is also possible to postulate that there is a positive feedback mechanism in such a way that the amount of expressed BDNF or the amount of expressed BDNF receptors would be enhanced by repetition [20,24]. However, the reverse is also possible: previous authors have reported a negative feedback mechanism in the TrkB surface expression in response to prolonged exposure to BDNF [5]. Experiments testing possible feedback mechanisms from this point of view in the present system are currently underway.

As to the relationship between the signaling pathways of PKA and BDNF, a ‘gating’ effect is presumed. In frog neuromuscular junctions, blockade of PKA abolished an acute effect of BDNF to increase the frequency and amplitude of miniature endplate currents, whereas PKA activator or inhibitor alone had no effect [4], suggesting that PKA activity opens the gate of BDNF signaling pathway. In cultured hippocampal slice, the blockade of PKA also abolished the effect of a 12–24 h exposure to BDNF to increase the expression of synaptotagmin, a species of synaptic vesicle protein [22]. In the dissociated cell culture of hippocampal neurons, BDNF applied for 24 h together with Sp-cAMPS accelerated the outgrowth of dendritic spines, whereas Sp-cAMPS had no effect by itself [8]. Although the interaction of signaling pathways of PKA and BDNF in the present phenomenon seems distinct from that of the above phenomena in the point that PKA activation is effective by itself, it is probable that PKA signal may act in two ways, one to induce the release of BDNF and the other to gate the BDNF signal.

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