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Brain-derived Neurotrophic Factor Induces Rapid and Transient Release of Glutamate through the Non-exocytotic Pathway from Cortical Neurons*

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Nobuyuki Takei‡§¶, Tadahiro Numakawa¶, Shunji Kozaki‖, Naoto Sakai¶, Yasuhisa Endo‡, Masami Takahashi, and Hiroshi Hatanaka¶**

*From the ‡Department of Applied Biology, Kyoto Institute of Technology, Sakyo, Kyoto 606, ¶Division of Protein Biosynthesis, Institute for Protein Research, Osaka University, Suita, Osaka 565, ‖Department of Veterinary Science, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 593, and **Mitsubishi Kasei Institute for Life Sciences, Machida, Tokyo 194, Japan*

There is increasing interest in the involvement of neurotrophins in neural transmission and plasticity. Thus, we investigated the effects of brain-derived neurotrophic factor (BDNF) on glutamate release from cortical neurons. Treatment of cultured cortical neurons with BDNF induced rapid and transient release of glutamate. This effect was suggested to be mediated by TrkB activation because K252a inhibited the release of glutamate and BDNF phosphorylated TrkB within 30 s. BDNF-induced glutamate release was observed even when using Ca^{2+}-free assay buffer but was inhibited by BAPTA-AM, a cell-permeable Ca^{2+} chelator. Therefore, BDNF-induced glutamate release was independent of extracellular Ca^{2+} but dependent on intracellular Ca^{2+}. Because normal neurotransmitter release is exocytotic, the involvement of the exocytotic pathway in BDNF-induced glutamate release was examined. As botulinum toxin is known to cleave exocytosis-associated proteins, thereby inhibiting exocytosis, it was applied to neurons prior to the release assay. Although botulinum toxin B cleaved VAMP2 and inhibited Ca^{2+}-triggered glutamate release, it did not inhibit the BDNF-induced release of glutamate. These results strongly suggested that BDNF induces rapid and transient release of glutamate from cortical neurons through a non-exocytotic pathway.

Neurotrophins are the best characterized neurotrophic factors promoting differentiation, maturation, and survival of various types of neurons in both central and peripheral nervous systems (1, 2). In addition to their roles in the long term developmental processes, there is increasing interest in the involvement of neurotrophins in neural transmission and synaptic plasticity (3–6). It has also been reported that neurotrophins themselves are secreted in response to depolarizing stimuli such as high K^+ or glutamate, as well as constitutive secretion (7, 8). These findings strongly support the hypothesis that neurotrophins are involved in activity-dependent neural plasticity. In cultured *Xenopus* neuromuscular synapses, BDNF[1] and neurotrophin-3 elevated the frequency of spontaneous synaptic currents and enhanced the amplitude of evoked synaptic currents within 10 min (9). Similar phenomena were observed for the effects of BDNF and neurotrophin-4 on the synapses in hippocampal culture. BDNF and neurotrophin-4 rapidly increased both the frequency of spontaneous synaptic currents and the amplitude of evoked synaptic currents (10). Furthermore, nerve growth factor and BDNF have been reported to augment the evoked release of acetylcholine and glutamate from hippocampal synaptosomes (11). These reports indicated an effect of neurotrophins on neurotransmitter release. In addition to these results, neurotrophins induced long lasting potentiation of neurotransmission (12) or enhanced long term potentiation (13) in hippocampal slices. Although several electrophysiological studies have been performed, the molecular basis of the presynaptic modulation of neurotransmitter release by neurotrophins is still unknown. The first step of neural transmission is regulated, stimulation-induced release of neurotransmitters. Thus, we tested the effects of BDNF on glutamate release through the activation of TrkB from cultured cortical neurons by measuring levels of the transmitter itself. In addition, we also analyzed the mechanisms of the release using botulinum toxin. Here, we demonstrated that BDNF induced glutamate release by a pathway independent of exocytosis.

EXPERIMENTAL PROCEDURES

Materials—BDNF was a kind gift from Regeneron Pharmaceutical Co. Anti-TrkB monoclonal antibody was kindly supplied by Dr. S. Koizumi (Novartis Pharma K. K.). Anti-VAMP2 antibody was prepared as described previously (14), and anti-phosphotyrosine antibody was purchased from Transduction Lab.

Cell Culture—The method of primary culture of cortical neurons was essentially as described previously (15). Briefly, cerebral cortices were removed from 17-day-old embryonic rat fetuses and dissociated using trypsin/DNase I. Neurons were seeded at a density of 5×10^5 cells/cm^2 in culture dishes and cultivated in minimum essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.). Medium was changed every 3 or 4 days. After 7–10 days, cells were used for release assay and collected for immunoprecipitation or Western blot analysis.

Measurement of Amino Acid Transmitters—Glutamate release from cultured neurons was determined essentially as described previously (16). Briefly, neurons were washed seven times with assay buffer and Ringer's solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5.5 mM glucose. High K^+ solution contained 50 mM KCl, and Ca^{2+}-free solution contained no $CaCl_2$. The assay buffer was

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§ To whom correspondence and reprint requests should be addressed: Dept. of Molecular Neurobiology, Brain Research Institute, Niigata University, Asahimachi 1, Niigata 951-8585, Japan. Tel.: 81-25-227-0615; Fax: 81-25-227-0814; E-mail: nobtak@bri.niigata-u.ac.jp.

[1] The abbreviations used are: BDNF, brain-derived neurotrophic factor; BoTX, botulinum toxin; TTX, tetrodotoxin; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester; RIPA, radioimmune precipitation buffer.

changed every 1 min, collected into tubes on ice, and filtrated to remove cell debris. To make derivatives of amino acids detectable by fluorescence monitoring, samples were mixed with *o*-phthalaldehyde (4:1) and allowed to stand for 5 min at room temperature. Samples were then applied to high pressure liquid chromatography and analyzed using a fluorescence monitor (excitation 340 nm, emission 445 nm).

Immunoprecipitation—For TrkB immunoprecipitation, cells were washed three times with 1 mM $NaVO_4$ containing TN buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl), lysed in radioimmune precipitation buffer (TN buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate), and incubated at 4 °C on a mixing plate for 3 h. After centrifugation, the supernatant was mixed with protein G-anti-TrkB antibody conjugate and incubated for 4–5 h at 4 °C on a mixing plate. The conjugate was prepared for incubation in radioimmune precipitation buffer for 3 h and washed 5 times with radioimmune precipitation buffer. The protein G-anti-TrkB-antigen complex was washed 5 times with radioimmune precipitation buffer and finally SDS sample buffer.

Electrophoresis and Western Blotting—SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described previously (16). Cells were collected and sonicated in TN buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl), and protein concentration was determined. Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were exposed to the first antibody (anti-phosphotyrosine or anti-VAMP2), washed, and then exposed to horseradish peroxidase-conjugated anti-mouse IgG (Cappel, 1:2000). Peroxidase activity was visualized on x-ray film after reaction with ECL reagent (Amersham Pharmacia Biotech).

RESULTS

BDNF Induces Glutamate Release from Cortical Neurons—Treatment of primary cultured cortical neurons with BDNF for 1 min induced a rapid and transient increase in glutamate release (Fig. 1*A*). This was similar to the high K^+-evoked release of glutamate (Fig. 1*B*), except that the amount and rate of release in response to BDNF were both less than those induced by high K^+. BDNF-induced glutamate release occurred slightly more slowly than that induced by high K^+. To determine whether neurons or astrocytes released glutamate in response to BDNF, we examined the glutamate release from pure astrocyte cultures. Although astrocytes spontaneously released a considerable amount of glutamate, BDNF did not increase the release (Fig. 1*C*). We also confirmed that glutamatergic neurons in the cultured cortical cells expressed TrkB using a double staining technique.[2] These results, combined with the observation that astrocytes do not express full-length TrkB, indicated that BDNF acts on glutamatergic neurons directly and triggers glutamate release.

BDNF induced glutamate release in a dose-dependent manner. Fig. 2 represents the ratio of BDNF-induced glutamate release. The effect was observed at a dose of 10 ng/ml and was saturated at 100 ng/ml.

BDNF Induces Glutamate Release through the Activation of TrkB—The involvement of TrkB activation in this rapid and transient response was also examined. As shown in Fig. 3*A*, pretreatment with K252a completely blocked the BDNF-induced release of glutamate but did not have any effect on the basal release. Furthermore, the tyrosine phosphorylation of TrkB, *i.e.* activation of the receptor, was observed within 30 s in response to BDNF (Fig. 3*B*). This effect was blocked by K252a (Fig. 3*B*) under conditions identical to those used in the release assay.

In contrast to BDNF, nerve growth factor had no effect on glutamate release from cortical neurons (Fig. 3*C*). This was expected because of the absence or low level of expression of TrkA in the cortex. Thus, the involvement of p75 was also excluded. Taken together, these results indicated that BDNF induces the release of glutamate through activation of TrkB but not p75.

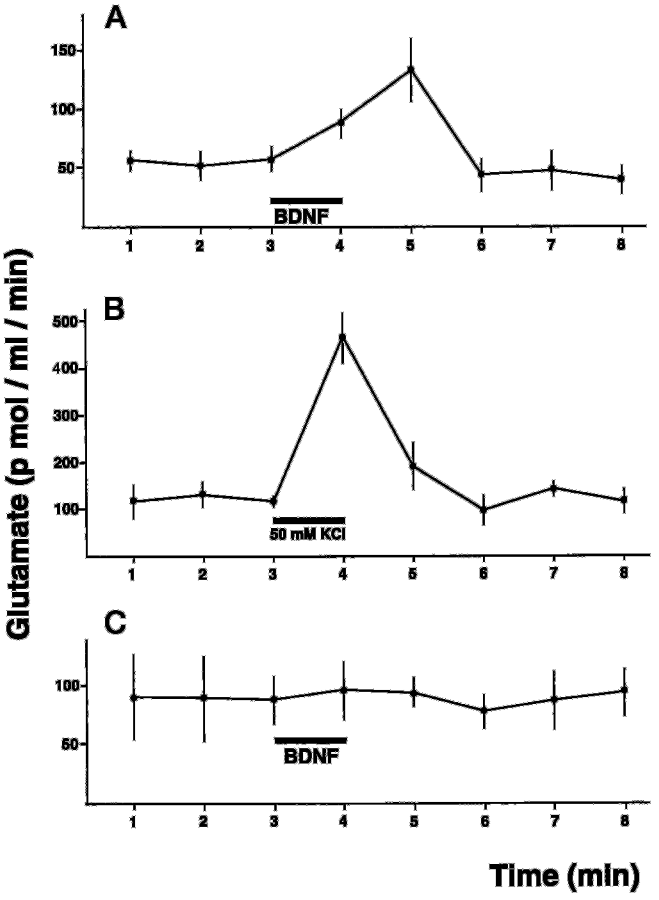

FIG. 1. **Time course of BDNF and high K^+-induced glutamate release.** Effects of BDNF (*A*, 100 ng/ml, $n = 16$) and high K^+ (*B*, 50 mM KCl, $n = 8$) for 1 min on the release of glutamate from cultured neurons are shown. *C*, effects of BDNF on the release of glutamate from astrocytes ($n = 4$) are shown. Values indicate mean ± S.D.

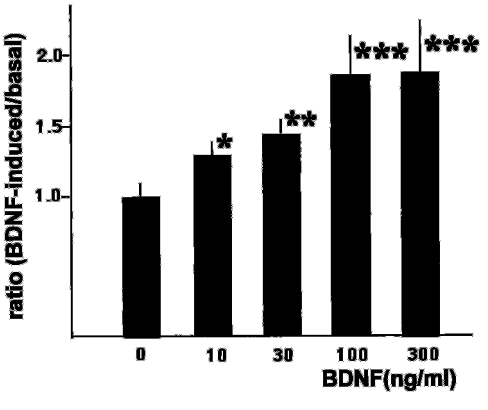

FIG. 2. **The effects of different doses of BDNF on glutamate release from cortical neurons in culture.** *Bars* indicate the ratio (BDNF-induced/basal) of glutamate release. Values indicate mean ± S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

BDNF Induced Glutamate Release Dependent on Intracellular but Not Extracellular Ca^{2+}—Next, Ca^{2+} dependence was investigated. In Ca^{2+}-free Ringer's solution, BDNF still induced glutamate release in a similar manner to that under normal conditions in the presence of 1.8 mM Ca^{2+} (Fig. 4). The same result was obtained when EGTA was added to the Ringer's solution (data not shown). Although the amount of glutamate released was decreased under both basal conditions and

[2] N. Takei, T. Numakawa, and H. Hatanaka, unpublished observation.

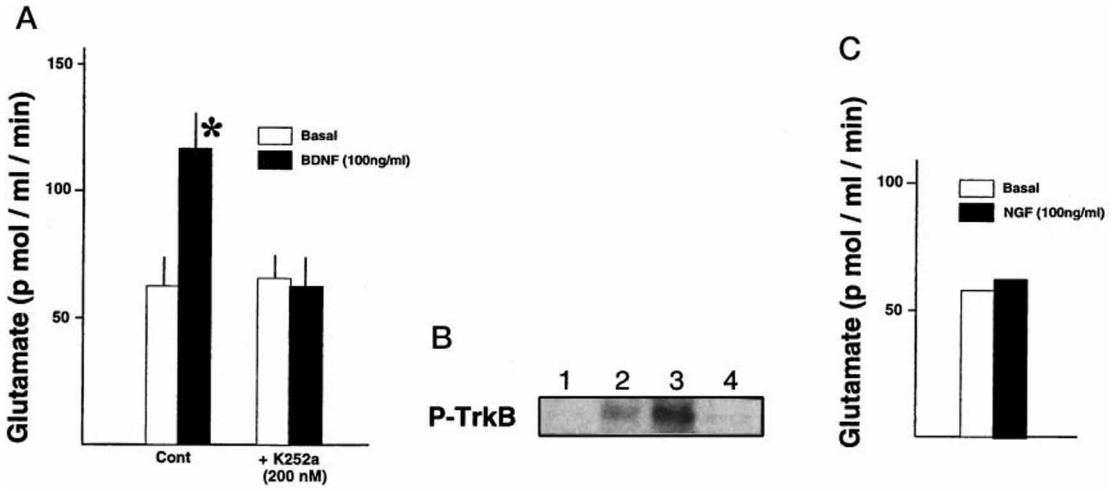

FIG. 3. **The involvement of TrkB activation on BDNF-induced glutamate release.** *A*, effects of K252a on BDNF-induced glutamate release ($n = 6$). Cells were pretreated with K252a (200 nM) for 20 min before the assay. Values indicate mean ± S.D. *Cont*, control. *B*, rapid phosphorylation of TrkB by BDNF. Neurons were lysed and immunoprecipitated by anti-TrkB antibody followed by Western blotting with anti-phosphotyrosine antibody. *Lane 1*, control; *lane 2*, 30 s; *lane 3*, 1 min after stimulation; and *lane 4*, 1 min + K252a under the same conditions as used for the release assay. Concentration of BDNF is 100 ng/ml in both experiments (*A* and *B*). *C*, effects of nerve growth factor (100 ng/ml) on the release of glutamate. Values indicate mean ($n = 2$). *, $p < 0.001$.

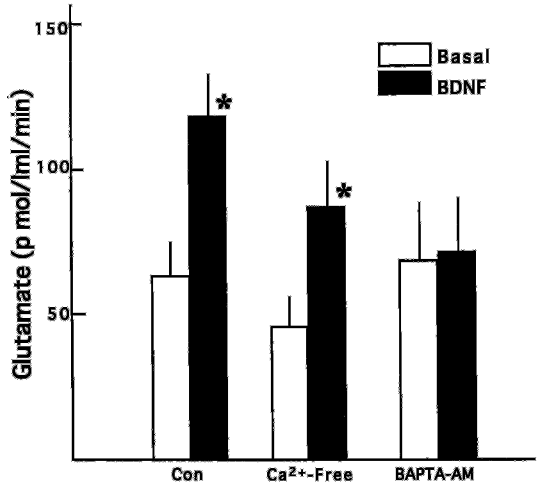

FIG. 4. **Ca^{2+} dependence of BDNF-induced glutamate release.** Ca^{2+}-free indicates Ringer's solution containing no $CaCl_2$, and BAPTA-AM indicates preincubation with this agent for 30 min before the assay ($n = 6$). *Con*, control. *, $p < 0.001$.

in BDNF-treated cells, the ratio of basal to induced release was unchanged. This decrease may have been because of calcium-dependent spontaneous exocytosis. In contrast, the cell membrane-permeable calcium chelator, BAPTA-AM, completely blocked BDNF-induced release. These results indicated that BDNF-induced glutamate release is independent of the extracellular calcium level but is dependent on the intracellular calcium level.

BDNF-induced Glutamate Release by a Non-exocytotic Pathway—We examined whether an exocytotic pathway was involved in the BDNF-induced glutamate release using botulinum toxin B (BoTX/B), which cleaves VAMP2 and inhibits exocytosis. Pretreatment of cortical neurons with BoTX/B for 8–12 h caused complete inhibition of ionomycin-evoked glutamate release (Fig. 5*B*). Ionomycin is a Ca^{2+}-ionophore and is known to elicit typical Ca^{2+}-triggered exocytosis. BDNF-induced glutamate release was affected little by BoTX/B (Fig. 5*A*). To confirm the effects of BoTX/B on the cleavage of VAMP2 in cultured neurons, Western blotting was performed after the release assay. As shown in Fig. 5*C*, BoTX/B markedly reduced the VAMP2 protein levels. Similarly, BoTX/A, which cleaves synaptosome-associated membrane protein-25 (SNAP-25), and the combination of BoTX/A and BoTX/B did not affect BDNF-induced glutamate release (data not shown). These results strongly suggested that BDNF-induced glutamate release is independent of the normal exocytotic pathway.

BDNF-induced Glutamate Release Was Inhibited by TTX—We then tested the Na^+ dependence of BDNF-induced glutamate release using TTX, a blocker of sodium channels. TTX was found to inhibit the glutamate release induced by BDNF (Fig. 6), indicating that BDNF-induced glutamate release is sodium-dependent. In summary, BDNF induced glutamate release through activation of TrkB. This effect was dependent on intracellular but not extracelluar Ca^{2+}, was non-exocytotic, and was sodium-dependent.

DISCUSSION

The main findings of this study were that BDNF induces glutamate release through the activation of TrkB and that its mechanism is non-exocytotic. BDNF induced rapid and transient release of glutamate from cortical neurons but not from astrocytes in culture. We also confirmed that glutamatergic neurons express TrkB in culture by immunocytochemistry.[2] This result is in agreement with the observations of postsynaptic events as revealed by electrophysiological and optical recording studies. Electrophysiological recording studies showed that BDNF rapidly increased the frequency of spontaneous synaptic currents in hippocampal neurons suggesting glutamate release from presynapses (10). We reported that synchronized oscillation of Ca^{2+}, which is thought to reflect synaptic transmission, was potentiated by BDNF in hippocampal (17) and cortical neurons.[3] The synchronized Ca^{2+} oscillation potentiated by BDNF was blocked by glutamate antagonist (17), suggesting that BDNF induced glutamate release. These pre- and postsynaptic reactions induced by BDNF suggest that BDNF is a physiological messenger in glutamatergic synapses. BDNF-induced glutamate release was inhibited by K252a, a Trk kinase inhibitor, and phosphorylation of TrkB was observed within 30 s. In addition, TrkB-IgG also blocked BDNF-induced glutamate release (data not shown). Nerve growth factor, which does not phosphorylate TrkB, had no effect on glutamate release in cortical cultures. Although the

[3] N. Sakai and H. Hatanaka, unpublished observation.

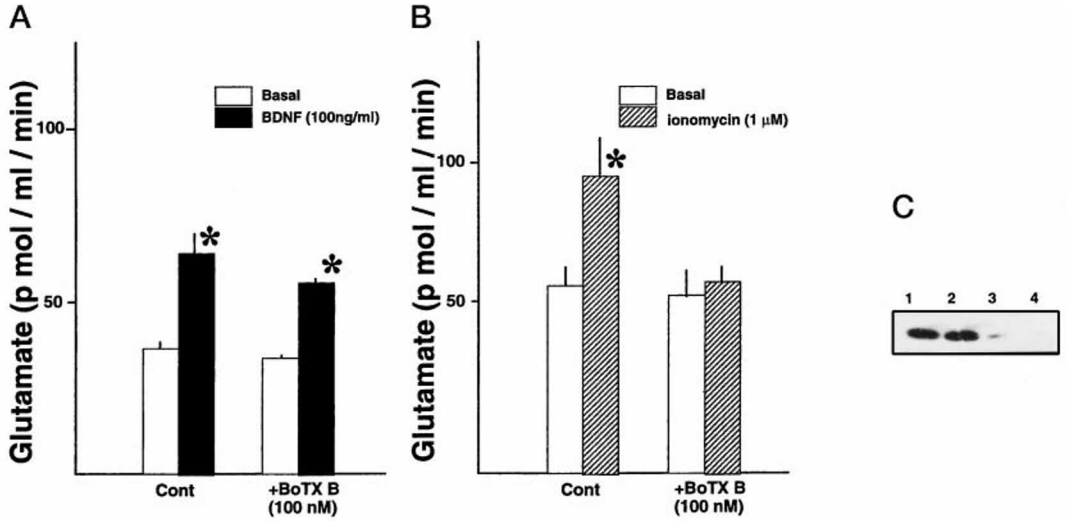

FIG. 5. **The mechanisms of BDNF-induced glutamate release.** Effects of BoTX/B on BDNF- induced (*A*) or ionomycin-induced (*B*) glutamate release ($n = 8$) are shown. Cultures were pretreated with BoTX/B (1 mM) for 8–12 h. Values indicate mean ± S.D. *Cont*, control. *C*, Western blotting analysis of VAMP2 to confirm the cleavage effect of BoTX/B. *Lanes 1* and *2*, control; *lanes 3* and *4*, BoTX/B-treated. *Lanes 1* and *3* are samples from ionomycin experiments, and *lanes 2* and *4* are from BDNF experiments. *, $p < 0.001$.

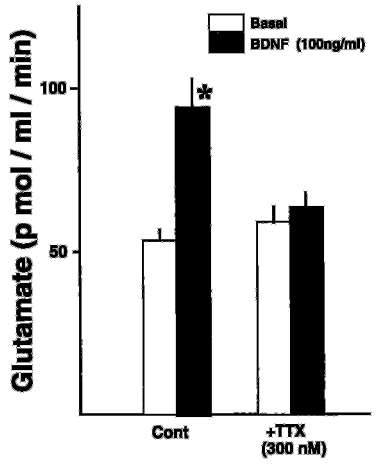

FIG. 6. **Effects of TTX on BDNF-induced glutamate release.** TTX (300 nM) was present throughout the assay from the washing step. Values indicate mean ± S.D. ($n = 8$). *Cont*, control. *, $p < 0.001$.

dose of BDNF seems relatively high compared with its affinity to TrkB, the effect of BDNF on glutamate release is dose-dependent. The dose shift may be explained by the experimental procedures. There were no proteins such as bovine serum albumin to prevent the adsorption of BDNF to pipette tips and culture dishes in this assay buffer, so the actual amount of BDNF that acted on the neurons may have been reduced. These results indicated that BDNF induces the release of glutamate through activation of TrkB.

Normal, regulated release of neurotransmitters produces Ca^{2+}-dependent exocytosis (18–20), and neurotrophins increase Ca^{2+} levels in neurons (21, 22). Therefore, Ca^{2+} dependence was then investigated. BDNF-induced glutamate release was independent of the extracellular calcium level but dependent on the intracellular calcium level. These observations suggest two possibilities: 1) Ca^{2+} release from the intracellular stores may be required to generate a Ca^{2+} transient; or 2) a specific level of free Ca^{2+} may be necessary as a basal condition.

We examined whether an exocytotic pathway was involved in the BDNF-induced glutamate release using BoTX/B. BoTX/B is known to cleave VAMP2 (23), a synaptic vesicle membrane protein that forms a component of the SNARE complex (18, 19, 24), thereby inhibiting exocytosis (18, 19, 23). Whereas ionomycin-evoked glutamate release, which is known to elicit the Ca^{2+}-triggered typical exocytosis, was inhibited completely by BoTX/B, BDNF-induced glutamate release was not affected by this treatment. This result strongly suggested that BDNF-induced glutamate release is independent of the normal exocytotic pathway mediated by SNARE complexes (18, 19, 24).

There is another pathway of glutamate release distinct from exocytosis known as reverse transport through glutamate transporters (25, 26). The neuronal glutamate transporter EAAC1 has been cloned (27) and was reported to be expressed on glutamatergic neurons of the cerebral cortex and hippocampus (28). The glutamate transporter usually serves as an uptake carrier removing glutamate that has been released into the synaptic cleft under normal conditions. However, when the extracellular K^+ level or intracellular Na^+ level is high, the transporter works in the reverse direction (26). Indeed, high K^+-induced glutamate release was not completely inhibited by BoTX under our culture conditions (data not shown). Thus, we tested the Na^+ dependence of BDNF-induced glutamate release using TTX, a blocker of sodium channels. TTX was found to inhibit the glutamate release induced by BDNF. Although the result of the TTX experiment should be discussed carefully, in the case of neurotransmitter release independent of extracellular Ca^{2+} but dependent on Na^+, it has been suggested that increases in intracellular Na^+ concentration, rather than changes in membrane potential, are involved in inducing the release of transmitters by the mechanism of reverse transport (25). Thus, in our study it was likely that TTX inhibited BDNF-induced release of glutamate by blocking the increase of Na^+ concentration and not through inhibition of synaptic transmission.

These results suggest that BDNF increases the release of glutamate through reverse transport. Alternatively, BDNF may increase the uptake of glutamate into synaptic vesicles resulting in high vesicular glutamate concentration. In this case, a quantum of glutamate is increased; thus BDNF elevates the basal release. However, the electrophysiological data ran counter to this hypothesis. Neurotrophins increase the frequency of miniature excitatory postsynaptic currents without affecting their amplitude (10). This suggests that the effect is not attributable to an increase in quantum. BDNF also induced dopamine release from mesencephalic neurons via the TrkB

pathway (29). It is not clear whether the same mechanism is involved in both responses, as there have been no reports concerning Ca^{2+} and Na^{+} dependence or exocytosis. However, catecholamine is also released from the cytosolic pool via transporters (25). In the case of acetylcholine as reported by Lohof *et al.* (9), it is again not clear whether the same mechanisms are involved, as the time course is different and there is no evidence of reverse uptake of acetylcholine. However, there have been reports of Ca^{2+}-independent and Na^{+}-dependent release of acetylcholine (25).

Here, we demonstrated that BDNF induces rapid and transient release of glutamate from cortical neurons through the activation of TrkB. This is a presynaptic event corresponding to the physiological responses observed in postsynapses reported previously (9, 10, 12, 13, 17). BDNF-induced release of glutamate is unusual in that it occurs via a non-exocytotic mechanism. We suggest that this glutamate release occurs via reverse transport through the glutamate transporter, although confirmation is still required by direct evidence, possibly through the use of knock-out mutants. Transmitter release through the non-exocytotic pathway induced by physiological stimulation such as BDNF may be a novel mechanism of synaptic transmission. It has been reported that the expression (30) and release (7) of neurotrophins are modulated by glutamatergic transmission. In addition, we reported previously (16) that long term treatment with BDNF up-regulates the exocytotic machinery and transmitter release. Therefore, it is possible to envisage a feed forward enhancement of synaptic transmission and subsequent strengthening of the synaptic connections by BDNF and glutamate.

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