ACS chemical biology

LETTER

Cell-Based Fluorescent Indicator To Visualize Brain-Derived Neurotrophic Factor Secreted from Living Neurons

Takahiro Nakajima[†], Moritoshi Sato[‡,§], Naoko Akaza[†], and Yoshio Umezawa[†,*]

[†]Department of Chemistry, School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, [‡]Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Meguro-bu, Tokyo 153-8902, Japan, and [§]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan

ABSTRACT Brain-derived neurotrophic factor (BDNF) is a polypeptide that is secreted from neurons. Although there is mounting evidence that BDNF regulates neuronal development and synaptic plasticity, BDNF secretion has remained unclear due to lack of appropriate methods for the analysis of its dynamics. To visualize BDNF secretion from neurons, here we have developed a cell-based fluorescent indicator for BDNF. We showed that the present cell-based fluorescent indicator, named "Bescell", has high selectivity to BDNF and detects picomolar concentrations of BDNF (detection limit of 60 pM). Bescell has visualized endogenous BDNF secreted from hippocampal neurons. It thus provides a powerful tool for the analysis of BDNF secretion from living neurons.

*Corresponding author, umezawa@chem.s.u-tokyo.ac.jp.

Received for review July 12, 2007 and accepted May 2, 2008.

Published online May 30, 2008

10.1021/cb800052v CCC: $40.75

Cells secrete a wide variety of biological substances that regulate cellular functions, including growth factors, cytokines, hormones, and neurotransmitters. Brain-derived neurotrophic factor (BDNF) is a polypeptide that is secreted from neurons. BDNF plays a key role in the regulation of growth and survival of neurons (*1*). Decreased expression of BDNF is involved in pathophysiology of the brain, including Alzheimer's disease and Parkinson's disease (*2*). In addition to the long-term effects, BDNF contributes to a rapid modulation of neuronal functions such as activity-dependent synaptic plasticity, which is a cellular basis for learning and memory (*1*). For understanding the biological functions of BDNF, it is essential to reveal the dynamics of BDNF secretion from living neurons. However, convincing methods are not available that provide the spatiotemporal information about BDNF secretion.

BDNF has so far been analyzed by enzyme-linked immunosorbent assay (ELISA) as well as other growth factors, cytokines, hormones, and neurotransmitters (*3*). However, because ELISA is a destructive method, it provides no spatiotemporal information about BDNF secretion. In the present study, we have developed a cell-based indicator that detects picomolar concentrations of BDNF with fluorescence readout. Using the present cell-based fluorescent indicator, named "Bescell" (BDNF sensor cell), we visualized BDNF secretion from hippocampal neurons.

RESULTS AND DISCUSSION

A cell-based indicator is a living cell that is engineered to have functional proteins for recognition of an analyte, for signal amplification and transduction, and for detection of the amplified signal. To develop Bescell, we expressed a chimeric receptor protein kinase and a fluorescent indicator for protein phosphorylation in a living cell (Figure 1). An extracellular domain of the chimeric receptor binds with BDNF at the surface of the cell-based indicator (Figure 1). The binding of BDNF triggers activation of an intracellular kinase domain of the chimeric receptor, which transduces the signal (Figure 1). The activation of the kinase domain is detected with the fluorescent indicator for protein phosphorylation (Figure 1). Bescell thus detects physiological concentrations of BDNF with fluorescence readout. We first constructed the chimeric receptor tyrosine kinase and the fluorescent indicator for protein phosphorylation, respectively.

We constructed the chimeric receptor tyrosine kinase that binds with BDNF and transduces the signal to its kinase activation in Bescell. Tropomyosin receptor kinase B (TrkB) is a receptor for BDNF that is located at the surface of cells (*4*). We used

acs chemical biology

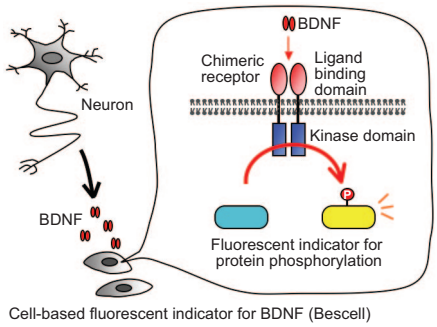

Figure 1. Schematic diagram of Bescell. BDNF secreted from neurons is received by the ligand binding domain of chimeric receptor at the surface of Bescell. The binding of BDNF induces dimerization of the chimeric receptor and thereby activates the kinase domain of the chimeric receptor. The activated kinase domain of the chimeric receptor phosphorylates the fluorescent indicator for protein phosphorylation in Bescell. The phosphorylated fluorescent indicator emits fluorescence signals. Bescell thus detects BDNF with fluorescence readout.

the extracellular BDNF binding domain of TrkB for the chimeric receptor (*5*). TrkB is a receptor tyrosine kinase. BDNF induces dimerization of TrkB and thereby activates the intracellular kinase domain of TrkB. Among receptor tyrosine kinases, EGF receptor (EGFR) is reported to show much higher kinase activity than others (*6*). Therefore, we constructed the chimeric receptor tyrosine kinase for BDNF using the intracellular kinase domain from EGFR (EGFR catalytic domain: ECat) and the extracellular BDNF binding domain of TrkB (BDNF binding domain: BBD). We expected that this chimeric receptor tyrosine kinase, named BBD-ECat, specifically binds to BDNF and exhibits high kinase activity.

We examined whether BBD-ECat is efficiently expressed and activated in living cells. We constructed cDNA encoding BBD-ECat as shown (Figure 2, panel a). The cDNA encoding BBD-ECat was transfected into cultured MCF-7 cells. The cells were stimulated with 1.1 nM BDNF for 15 min. After the semipurification of the receptor proteins from the cell lysate with WGA-agarose, immunoblot analysis was performed using anti-V5 antibody or anti-phosphotyrosine antibody (pY20). The lower part of panel b (Figure 2, panel b) shows that BBD-ECat is efficiently expressed in the cells. The upper part of panel b (Figure 2, panel b) shows that BBD-ECat is efficiently autophosphorylated upon stimulation with BDNF. These results indicate that the extracellular domain of BBD-ECat binds to BDNF and the intracellular domain of BBD-ECat is then activated as expected. We used BBD-ECat for Bescell.

We have previously reported a genetically encoded fluorescent indicator for visualizing protein phosphorylation based on fluorescence resonance energy transfer (FRET) (*7*). This approach has been applied to various protein kinases, including Akt (*8*), ERK (*9*), and Src (*10*). In the present study, we constructed a fluorescent indicator for Bescell based on the FRET approach. This fluorescent indicator is composed of a substrate domain that is phosphorylated by a protein kinase, a recognition domain that binds to the phosphorylated substrate domain, and two green fluorescent protein mutants (Figure 2, panel c). We used a 12 amino acids sequence derived from a substrate protein of EGFR known as Shc for the substrate domain (amino acids 236−247 of Shc). The substrate sequence contains tyrosine residue 239 that is phosphorylated by EGFR (*11*). For the recognition domain, we used Src homology 2 (SH2) domain of growth factor receptor-bound protein 2 (Grb2) (*11*). Upon phosphorylation of the substrate domain, the recognition domain binds to the phosphorylated substrate domain in the fluorescent indicator (Figure 2, panel c). This conformational change in the indicator induces FRET between the donor cyan fluorescent protein (CFP) and the acceptor yellow fluorescent protein (YFP) that are tethered to the N-terminus of the substrate domain and the C-terminus of the recognition domain, respectively (Figure 2, panel c). We named this FRET-based indicator "ECaus" (ECat-phocus).

We constructed cDNA encoding ECaus as shown (Figure 2, panel a). The cDNA encoding ECaus was transfected into Chinese hamster ovary cells expressing EGFR (CHO-EGFR cells). Immunoblot analysis shows that ECaus was expressed in CHO-EGFR cells and phosphorylated when the cells were stimulated with EGF (Figure 2, panel d). We observed the CHO-EGFR cells expressing ECaus with a fluorescence microscope. The fluorescence of ECaus was observed uniformly from the cytosol of the cells (Figure 2, panel e). When we added EGF to the cells, an emission ratio of CFP to YFP, which is a measure for FRET, immediately decreased and reached a plateau within 150 s (Figure 2, panels f and g). From these results, we confirmed that ECaus emits FRET signals when phosphorylated in living cells.

To construct Bescell, we expressed both BBD-ECat and ECaus in MCF-7 cells. BDNF binds to BBD-ECat at the surface of Bescell. The binding of BDNF induces the dimerization of BBD-ECat and activates the kinase domain of BBD-ECat inside Bescell. The activated BBD-ECat phosphorylates ECaus. The resultant conformational change in ECaus induces FRET signals through CFP and YFP. As expected, Bescell actually exhibited a FRET response when stimulated with BDNF (Figure 3, panel a). This response is much larger than that of the cell expressing TrkB and ECaus (Figure 3, panels a and b). The result demonstrates that using ECat in place of the kinase domain of TrkB is effective for highly sensitive detection of BDNF.

We examined the reversibility of Bescell. When BDNF dissociates from BBD-ECat, the activation of BBD-ECat is terminated. Then endogenous phosphatases in the cell can dephosphorylate ECaus, resulting in a reversible change in the conformation of ECaus. Indeed, the FRET response of Bescell recovered to the basal level after BDNF

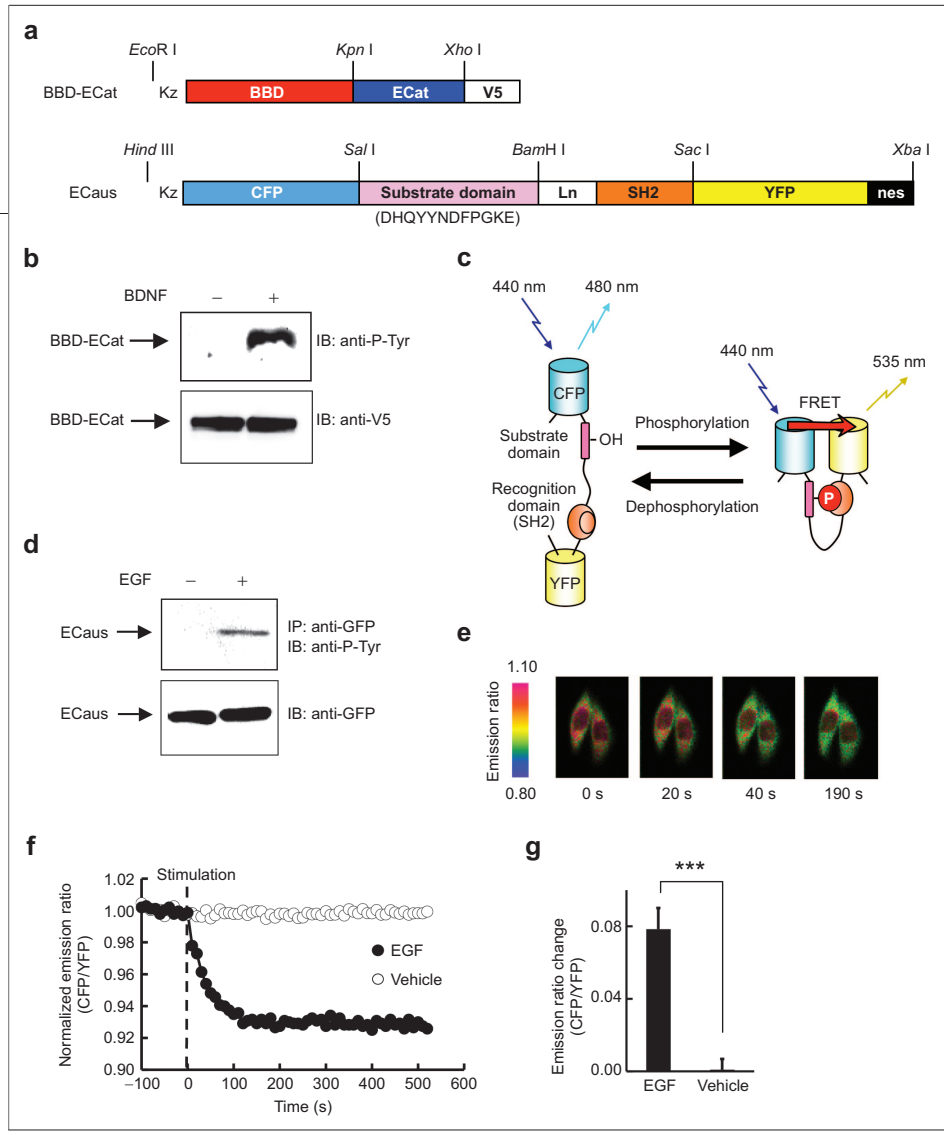

Figure 2. Construction of BBD-ECat and ECaus. a) Schematic representation of domain structure of BBD-ECat and ECaus: Kz, Kozak sequence; Ln, GGNNGNNGGNNGGNNGGNNGGNN; nes (nuclear export signal), LPPLERLTL. b) Autophosphorylation of BBD-ECat. MCF-7 cells expressing BBD-ECat were stimulated with 1.1 nM BDNF for 15 min at 25 °C. The whole-cell lysates were purified with WGA column, and immunoblot analysis was performed with anti-V5 antibody or anti-phosphotyrosine antibody (pY20). c) Principle of ECaus for visualizing protein phosphorylation by the kinase domain of EGFR (ECat). CFP and YFP are different-colored mutants of GFP. Upon phosphorylation of the substrate domain by ECat, the adjacent phosphorylation recognition domain binds with the phosphorylated substrate domain, which induces FRET from CFP to YFP. d) Phosphorylation of ECaus. CHO-EGFR cells expressing ECaus were stimulated with 100 ng mL^{-1} EGF for 15 min at 25 °C. The whole-cell lysates were immunoprecipitated with anti-GFP antibody, and immunoblot analysis was performed with anti-phosphotyrosine antibody (pY20) or anti-GFP antibody. e) Fluorescence imaging of ECaus upon EGF stimulation. Pseudocolor images of the CFP/YFP emission ratios before (time 0 s) and at 20, 40, and 190 s after the addition of 100 ng mL^{-1} EGF. f) Representative time courses of the CFP/YFP emission ratios from the cytosol of the cells expressing ECaus. At time 0 s (dashed line), the cells were stimulated with 100 ng mL^{-1} EGF or vehicle. g) Averaged emission ratio change with standard deviations of the cells expressing ECaus when stimulated with EGF (n = 5) or vehicle (n = 5). Triple asterisks indicate $p < 0.001$ (t test).

was removed (Figure 3, panels c and d). The result indicates that Bescell is the reversible indicator for BDNF.

We next examined the selectivity of Bescell to biological substances that neurons secrete. Nerve growth factor (NGF) is a neurotrophic factor that binds to TrkA but not to the BDNF receptor TrkB (*5*). Upon addition of NGF, Bescell showed no response as expected (Figure 3, panels e and f). Next we added acetylcholine (Ach) or L-glutamate (L-Glu), which are major neurotransmitters that neurons release. Also, no FRET response was observed to these neurotransmitters (Figure 3, panels e and f). These results indicate that Bescell is highly selective to BDNF.

To examine the dose−response curve, we added various concentrations of BDNF to Bescell. Figure 3, panel g, shows the averaged response of Bescell to each BDNF concentration. The result indicates that it detects picomolar concentrations of BDNF (detection limit of 60 pM).

In addition to BDNF, neurotrophin-4 (NT-4) and neurotrophin-3 (NT-3) are also endogenous ligands for TrkB receptor. We added various concentrations of NT-4 and NT-3 to Bescell, respectively. It showed only a slight response to NT-3 (Figure 3, panel g). This is due to the weak binding affinity of NT-3 to the extracellular domain of TrkB (*12*). On the other hand, NT-4 exhibited a comparable dose−response curve to that of BDNF (Figure 3, panel g). This is because the binding affinity of NT-4 to the extracellular domain of TrkB is equivalent to that of BDNF (*12*). However, the expression level of NT-4 is 100 times lower than that of BDNF in hippocampal neurons (*13*). Thus, both NT-3 and NT-4 are not considered to interfere with the response of Bescell to BDNF that is secreted from hippocampal neurons.

We cocultured Bescell with hippocampal neurons to visualize BDNF secretion from the neurons. We observed Bescell adjacent to a hippocampal neuron with a fluorescence microscope (Figure 4, panel a). We added 10 μM glutamate, an excitatory neurotransmitter, to activate the hippocampal neurons. Immediately after the stimulation of hip-

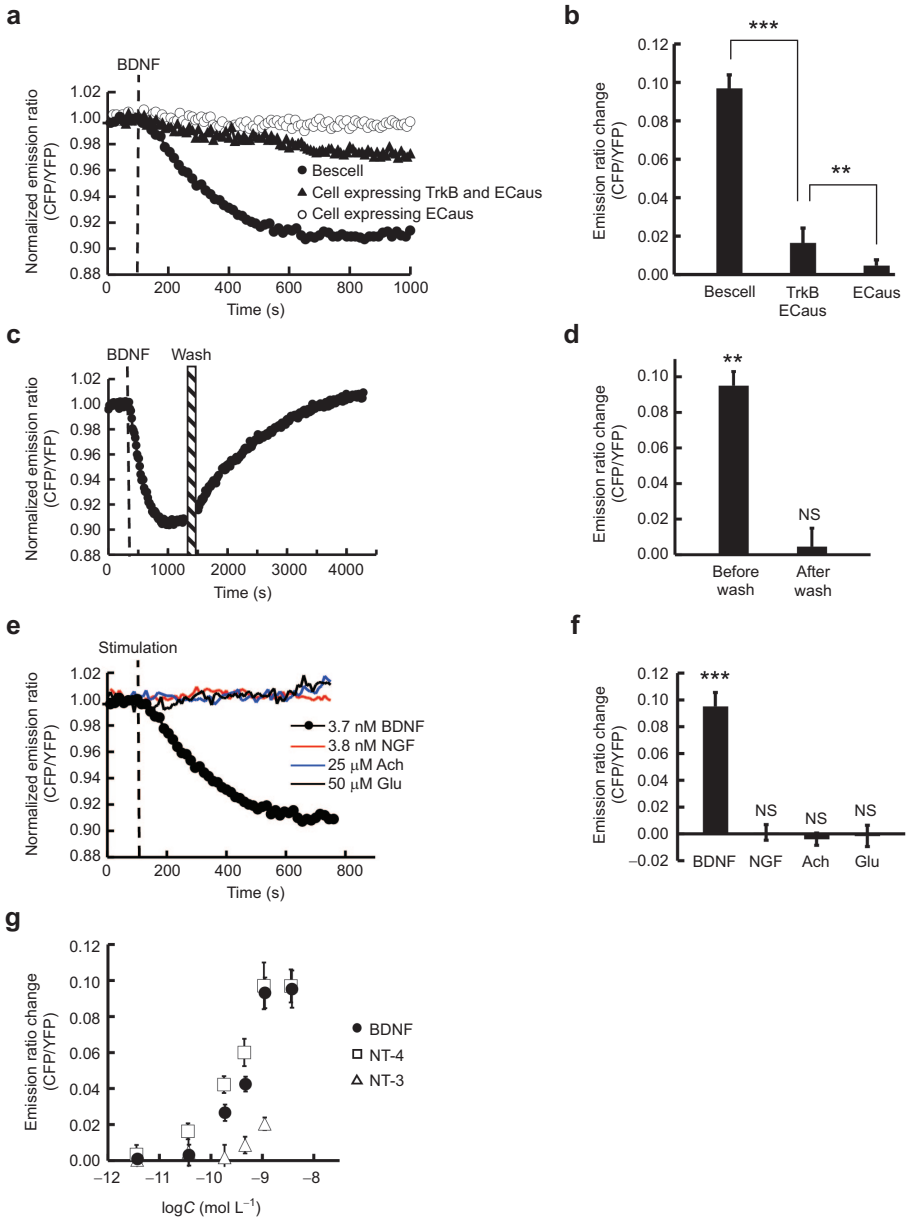

Figure 3. Development and characterization of Bescell. a) Response of Bescell for BDNF. Representative time courses of the CFP/YFP emission ratios from Bescell, MCF-7 cell expressing TrkB and ECaus, or MCF-7 cell expressing ECaus. At the time indicated with the dashed line, 3.7 nM BDNF was added to the cells. b) Averaged emission ratio change with standard deviations of Bescell ($n = 3$), MCF-7 cell expressing TrkB and ECaus ($n = 3$), or MCF-7 cell expressing ECaus ($n = 3$). Triple asterisks indicate $p < 0.001$ and double asterisks indicate $p < 0.01$ (t test). c) Reversibility of Bescell. Bescell was stimulated with 3.7 nM BDNF at the time indicated with the dashed line. After the response of Bescell reached a steady level, BDNF was washed out at the time indicated with the hatched bar. This result is a representative of three independent experiments. d) Averaged emission ratio change with standard deviations of before or after washout of BDNF ($n = 3$). Double asterisks indicate $p < 0.01$ and NS indicates not significant compared to before stimulation with BDNF (paired t test). e) Selectivity of Bescell. Representative time courses of FRET responses of Bescell when Bescell was stimulated with BDNF, NGF, Ach, or Glu. Each stimulation was done at the time indicated with the black dashed line. f) Averaged emission ratio change with standard deviations of Bescell stimulated with 3.7 nM BDNF ($n = 5$), 3.8 nM NGF ($n = 3$), 25 μM Ach ($n = 4$), or 50 μM Glu ($n = 3$). Triple asterisks indicate $p < 0.001$ and NS indicates not significant compared to before stimulation (paired t test). g) Dose−response curves of Bescell for BDNF, NT-4, and NT-3. The results are the means with standard deviations from five independent experiments.

pocampal neurons with glutamate, a gradual FRET response of Bescell was observed (Figure 4, panel b, and Supplementary Figure 1). The FRET response of it disappeared in the presence of a quenching anti-BDNF antibody (Figure 4, panels c and d).

The results indicate that Bescell detects BDNF that is secreted from hippocampal neurons. Its concentration was estimated to be 230 ± 70 pM from the dose−response curve of Bescell (Figure 3, panel g, and Figure 4, panel d). Also, because it exhibits the reversible response to BDNF (Figure 3, panel c), the gradual FRET response of Bescell (Figure 4, panel b) indicates that hippocampal neurons continuously secrete BDNF for about 500 s when stimulated with glutamate.

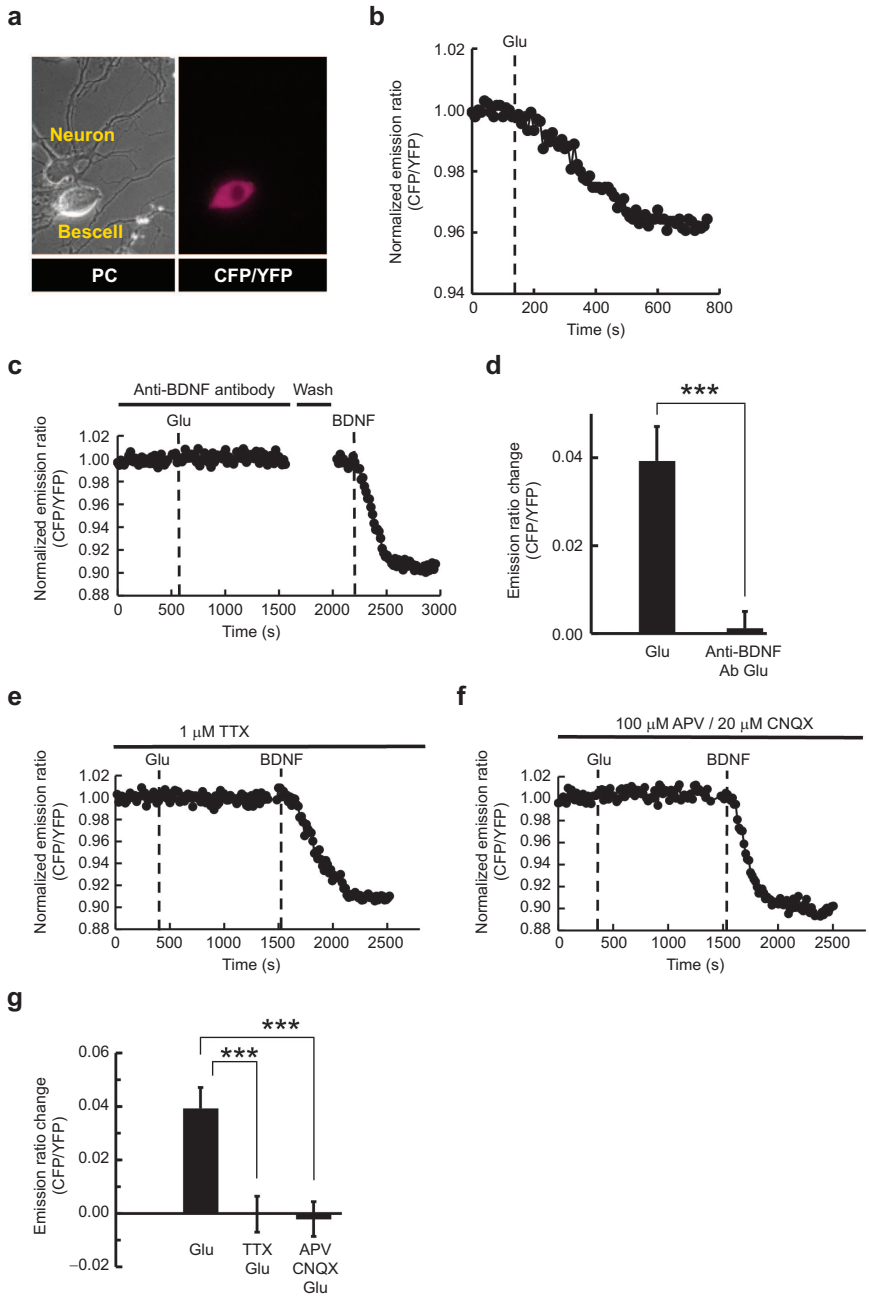

Figure 4. Visualization of BDNF secretion from hippocampal neurons. a) Phase contrast (left) and pseudocolor fluorescence ratio (right) images of cocultured hippocampal neurons and Bescell. b) Representative time course of a FRET response of Bescell located adjacent to the hippocampal neuron. The dashed line indicates the time when 10 μM Glu was added to the hippocampal neuron. c) Representative time course of a FRET response of Bescell when hippocampal neurons were incubated with a quenching antibody for BDNF. In the presence of 10 μg mL^{-1} anti-BDNF antibody, hippocampal neurons were stimulated with 10 μM Glu at the time as indicated with the first dashed line. After washout of the antibody, 3.7 nM BDNF was added at the time as indicated with the second dashed line. d) Averaged emission ratio change with standard deviations of Bescell when hippocampal neurons were stimulated with 10 μM Glu in the absence ($n = 5$) or presence ($n = 3$) of anti-BDNF antibody. Triple asterisks indicate $p < 0.001$ (*t* test). e) Representative time course of a FRET response of Bescell adjacent to the hippocampal neuron in the presence of TTX. The hippocampal neurons were incubated with 1 μM TTX during the observation. Ten micromolar Glu and 3.7 nM BDNF were added at the time as indicated with the dashed lines, respectively. f) Representative time course of a FRET response of Bescell in the presence of APV and CNQX. The hippocampal neurons were treated with 100 μM APV and 20 μM CNQX during the observation. Ten micromolar Glu and 3.7 nM BDNF were added at the time as indicated with the dashed lines, respectively. g) Averaged emission ratio change with standard deviations of Bescell when hippocampal neurons were stimulated with 10 μM Glu in the presence of TTX ($n = 4$) or APV and CNQX ($n = 3$). Triple asterisks indicate $p < 0.001$ compared to no inhibitor (*t* test).

Next we stimulated hippocampal neurons with glutamate in the presence of tetrodotoxin (TTX), which blocks voltage-gated Na^+ channels and thereby inhibits action potential generation. The TTX treatment resulted in disappearance of the FRET response of Bescell upon stimulation of hippocampal neurons with glutamate (Figure 4, panels e and g). Also we incubated hippocampal neurons with APV and CNQX, which are antagonists of the ionotropic glutamate receptors, NMDA receptor and AMPA receptor, respectively, to block glutamatergic synaptic transmission. This treatment with the antagonists blocked the FRET re-

sponse of Bescell (Figure 4, panels f and g). These results indicate that Bescell detects BDNF secretion from hippocampal neurons that is provoked in a neuronal activity-dependent manner. Thus, the present cell-based fluorescent indicator, Bescell, provides a powerful tool for the analysis of BDNF secretion from hippocampal neurons.

In the present study, we have developed a cell-based fluorescent indicator for BDNF based on a chimeric receptor tyrosine kinase and a FRET-based indicator for protein phosphorylation. The signal amplification by the enzymatic activity of the chimeric receptor allowed the detection of picomolar concentrations of BDNF. Bescell has actually visualized picomolar concentrations of BDNF secreted from hippocampal neurons after stimulation with glutamate. Because Bescell is much larger than synapse, the detected BDNF is considered to reflect the sum of BDNF diffused from many synapses in close proximity to Bescell. Thus, Bescell is a suitable method for visualization of BDNF diffused from neurons in a paracrine fashion.

Kojima *et al*. have previously developed a fusion protein of BDNF and GFP (BDNF-GFP) (*14*). They and other groups introduced BDNF-GFP into hippocampal neurons and visualized how BDNF-GFP is transported in axons and dendrites of the neurons (*14*–*16*). In contrast to this existing method, Bescell detects endogenous BDNF secreted out of neurons. It has allowed us to reveal to what extent and how endogenous BDNF is secreted from living hippocampal neurons.

BBD of the present chimeric receptor BBD-ECat plays a key role in the selective detection of BDNF in Bescell. The specific binding of BDNF with BBD induces the dimerization of BBD-ECat, which activates the kinase activity of ECat. A wide variety of peptide ligands including growth factors and cytokines also have their specific receptor tyrosine kinases or receptors associated with tyrosine kinases. The binding of ligand with its receptor induces the dimerization of the receptor tyrosine kinases. Therefore, replacement of BBD in the present chimeric receptor with ligand binding domains derived from the receptors may create chimeric receptors that lead to the development of cell-based fluorescent indicators for growth factors and cytokines such as EGF, PDGF, NGF, and interleukins.

METHODS

Plasmid Construction. To construct cDNAs of ECaus and BBD-ECat, fragment cDNAs of CFP, YFP, substrate domain, phosphorylation recognition domain, BBD, and ECat were generated by PCR and cloned into pBlueScript. All PCR fragments were sequenced with an ABI310 genetic analyzer (Applied Biosystems). The amino acid sequence of the substrate domain is DHQYYNDFPGKE, which is phosphorylated by EGFR (*11*). The phosphorylation recognition domain is derived from Grb2 (amino acid residues 58−153). The BBD is an extracellular domain of rat TrkB (amino acid residues 1−410). The ECat contains a transmembrane domain and an intracellular kinase domain of human EGFR (amino acid residues 628−1211). ECaus was subcloned into pcDNA3.1(+). BBD-ECat was subcloned into pcDNA3.1(+) His-V5-A to be tagged with V5 epitope tag.

Cell Culture and Transfection. CHO-EGFR cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin at 37 °C in 5% CO_2. Human breast adenocarcinoma cells (MCF-7 cells) were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin, 1 mM sodium pyruvate, and 0.1 mM MEM non-essential amino acids at 37 °C in 5% CO_2. Primary hippocampal neurons were prepared from Wistar rat embryos (embryonic day 17) and cultured in Neurobasal medium supplemented with 2% B-27 and 0.5 mM L-glutamine at 37 °C in 5% CO_2. Plasmid transfection was performed using FuGENE HD transfection reagent (Roche Applied Science). For the construction of Bescell, MCF-7 cells were transfected with the plasmids of BBD-ECat and ECaus at the ratio of 1:1. The transfected cells were plated 5−24 h post-transfection onto plastic culture dishes or glass-bottom dishes for immunoblot analysis or fluorescence imaging.

Imaging of Cells. Twenty-four hours after the transfection, the culture medium was replaced with a starvation medium (Ham's F-12 medium supplemented with 0.2% bovine serum albumin, 100 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin) in which the cells were incubated for 2−4 h at 37 °C in 5% CO_2. After the starvation, the medium was replaced with Hank's balanced salt solution. The cells were imaged at 25 °C on a Carl Zeiss Axiovert 200 microscope with a cooled CCD camera CoolSNAP HQ (Roper Scientific), controlled by MetaFluor (Universal Imaging). Upon excitation at 440 ± 10 nm, fluorescence images were obtained through a 480 ± 15 nm filter for CFP and 535 ± 12.5 nm filter for YFP with a 40× oil immersion objective (Carl Zeiss). The fluorescence intensities of CFP and YFP were measured from the cytosol of the cells. After baseline measurements, the cells were stimulated with neurotrophins or neurotransmitters. The emission ratio (CFP/YFP) was normalized to the baseline value. The added stimulants were not removed from glass-bottom dishes in all experiments, except for the washout experiments to examine the reversibility of Bescell. To examine a dose−response curve, Bescell was stimulated with 3.7 pM, 37 pM, 185 pM, 460 pM, 1.1 nM, or 3.7 nM BDNF. We added BDNF to the external solution of Bescell cultured in a glass-bottom dish, and then observed a FRET response of Bescell. Each Bescell was stimulated only once with each concentration of BDNF. The obtained change in the normalized emission ratio was plotted against each concentration of BDNF. The detection limit of Bescell was obtained from the concentration that gives a fluorescence signal equal to the background plus three times the standard deviation of the background.

Acknowledgment: This work has been supported by grants from the Japan Science and Technology Agency (JST), the Japan Society for the Promotion of Science (JSPS), the Takeda Science Foundation (to M.S.), and the Sumitomo Foundation (to M.S.).

Supporting Information Available: This material is available free of charge *via* the Internet.

REFERENCES

1. Lessmann, V., Gottmann, K., and Malcangio, M. (2003) Neurotrophin secretion: current facts and future prospects, *Prog. Neurobiol. 69*, 341–374.
2. Murer, M. G., Yan, Q., and Raisman-Vozari, R. (2001) Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease, *Prog. Neurobiol. 63*, 71–124.
3. Balkowiec, A., and Katz, D. M. (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons, *J. Neurosci. 22*, 10399–10407.
4. Middlemas, D. S., Lindberg, R. A., and Hunter, T. (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors, *Mol. Cell. Biol. 11*, 143–153.
5. Ultsch, M. H., Wiesmann, C., Simmons, L. C., Henrich, J., Yang, M., Reilly, D., Bass, S. H., and de Vos, A. M. (1999) Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC, *J. Mol. Biol. 290*, 149–159.
6. Sasagawa, S., Ozaki, Y., Fujita, K., and Kuroda, S. (2005) Prediction and validation of the distinct dynamics of transient and sustained ERK activation, *Nat. Cell Biol. 7*, 365–373.
7. Sato, M., Ozawa, T., Inukai, K., Asano, T., and Umezawa, Y. (2002) Fluorescent indicators for imaging protein phosphorylation in single living cells, *Nat. Biotechnol. 20*, 287–294.

8. Sasaki, K., Sato, M., and Umezawa, Y. (2003) Fluorescent indicators for Akt/protein kinase B and dynamics of Akt activity visualized in living cells, *J. Biol. Chem. 278*, 30945–30951.
9. Sato, M., Kawai, Y., and Umezawa, Y. (2007) Genetically encoded fluorescent indicators to visualize protein phosphorylation by extracellular signal-regulated kinase in single living cells, *Anal. Chem. 79*, 2570–2575.
10. Hitosugi, T., Sasaki, K., Sato, M., Suzuki, Y., and Umezawa, Y. (2007) Epidermal growth factor directs sex-specific steroid signaling through Src activation, *J. Biol. Chem. 282*, 10697–10706.
11. Sato, K., Gotoh, N., Otsuki, T., Kakumoto, M., Aoto, M., Tokmakov, A. A., Shibuya, M., and Fukami, Y. (1997) Tyrosine residues 239 and 240 of Shc are phosphatidylinositol 4,5-bisphosphate-dependent phosphorylation sites by c-Src, *Biochem. Biophys. Res. Commun. 240*, 399–404.
12. Naylor, R. L., Robertson, A. G., Allen, S. J., Sessions, R. B., Clarke, A. R., Mason, G. G., Burston, J. J., Tyler, S. J., Wilcock, G. K., and Dawbarn, D. (2002) A discrete domain of the human TrkB receptor defines the binding sites for BDNF and NT-4, *Biochem. Biophys. Res. Commun. 291*, 501–507.
13. Katoh-Semba, R., Ichisaka, S., Hata, Y., Tsumoto, T., Eguchi, K., Miyazaki, N., Matsuda, M., Takeuchi, I. K., and Kato, K. (2003) NT-4 protein is localized in neuronal cells in the brain stem as well as the dorsal root ganglion of embryonic and adult rats, *J. Neurochem. 86*, 660–668.
14. Kojima, M., Takei, N., Numakawa, T., Ishikawa, Y., Suzuki, S., Matsumoto, T., Katoh-Semba, R., Nawa, H., and Hatanaka, H. (2001) Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons, *J. Neurosci. Res. 64*, 1–10.
15. Kohara, K., Kitamura, A., Morishima, M., and Tsumoto, T. (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons, *Science 291*, 2419–2423.
16. Hartmann, M., Heumann, R., and Lessmann, V. (2001) Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses, *EMBO J. 20*, 5887–5897.