Supplemental Note 1: Specificity of Polyclonal Antibodies to BDNF.

We have examined the specificity of polyclonal antibodies against BDNF. We found that the N-20 antibody was slightly cross-reactive to an unidentified protein of ~55 kDa. Thus, we cannot exclude the possibility that some proportion of signals in immunostained neurons was derived from cross-reactivity of the antibody. To overcome this problem, we performed two independent sets of experiments using different polyclonal antibodies.

1. Overexpression of BDNF-pHluorin increased the N-20 antibody immunostaining signals of BDNF in cultured hippocampal neurons (supplemental Fig. 2).

2. As a complementary approach to overexpression, we examined whether knockdown of BDNF protein led to decreased immunostaining signals in cultured neurons. We constructed microRNA knockdown vectors with three different target sequences for BDNF (miR-BDNF1079, miR-BDNF1217 and miR-BDNF1360), and tested their effectiveness in HEK293 cells. We co-transfected HEK293 cells with a mixture of plasmids encoding one of the microRNAs, BDNF-pHluorin and GST-mCherry at a molecular ratio of 2:1:1. We then probed the Western blots of the HEK293 cell lysates with N-20 antibody, and reprobed them with anti-RFP antibody to examine the expression levels of GST-mCherry and thereby compensate the differences in transfection efficiency among experiments. We compared BDNF immunoreactivity under each condition with the control where microRNA knockdown vector for beta-galactosidase (miR-lacZ) was used instead. We found that miR-BDNF1360 was the most efficacious microRNA in reducing the signal of the pro- and mature form of BDNF-pHluorin to 9.2 ± 4.7% of a control in HEK293 cells (supplemental Fig. 1B). We noted that a non-specific signal at ~55 kDa, which was consistently observed as a weak, non-specific band in rat hippocampal neurons lysates (supplemental Fig. 1A, B and supplemental Fig. 4B), was not affected with any of the knockdown vectors, suggesting that gene silencing was specific to BDNF. We transfected cultured hippocampal neurons with miR-BDNF1360 or miR-lacZ, and immunostained them with N-20 antibody for the endogenous BDNF. Transfected neurons were identified by emerald GFP encoded by the knockdown vectors. We found that the immunostaining signals for endogenous BDNF were reduced in knockdown neurons but not in control

neurons (supplemental Fig. 1C). Diffuse residual signals in the BDNF knockdown neurons may be due to partial knockdown or surface-bound or endocytosed BDNF protein which had been secreted by un-transfected neighboring neurons, or non-specific staining originating from un-identified ~55 kDa protein.

3. Western blots of the wild-type mouse brain and rat hippocampal culture lysates using the N-20 antibody showed a specific ~15 kDa signal corresponding to the mature BDNF, which was absent in the brain lysate from BDNF knockout mice, in addition to the weak, nonspecific signals at ~55 kDa and ~65 kDa (supplemental Fig. 1A; see also supplemental Fig. 4B).

4. We also tested a custom-made polyclonal antibody to BDNF (provided by Dr. Ritsuko Katoh-Semba). The polyclonal antibody showed immunofluorescent signals similar to those found with the N-20 antibody, and the signals were again greatly reduced in BDNF-knockdown neurons (supplemental Fig. 1C). Using this antibody, we found that immunostaining signals of BDNF-containing vesicles were unchanged at the axon after 1 min TBS, but reduced after 3 min TBS. Immunoreactivity of BDNF at the dendrite was reduced when 1 or 3 min TBS was given. These changes were in parallel to those observed with the N-20 antibody (supplemental Fig. 9).

Supplemental Note 2: Expression and Processing of BDNF-pHluorin.

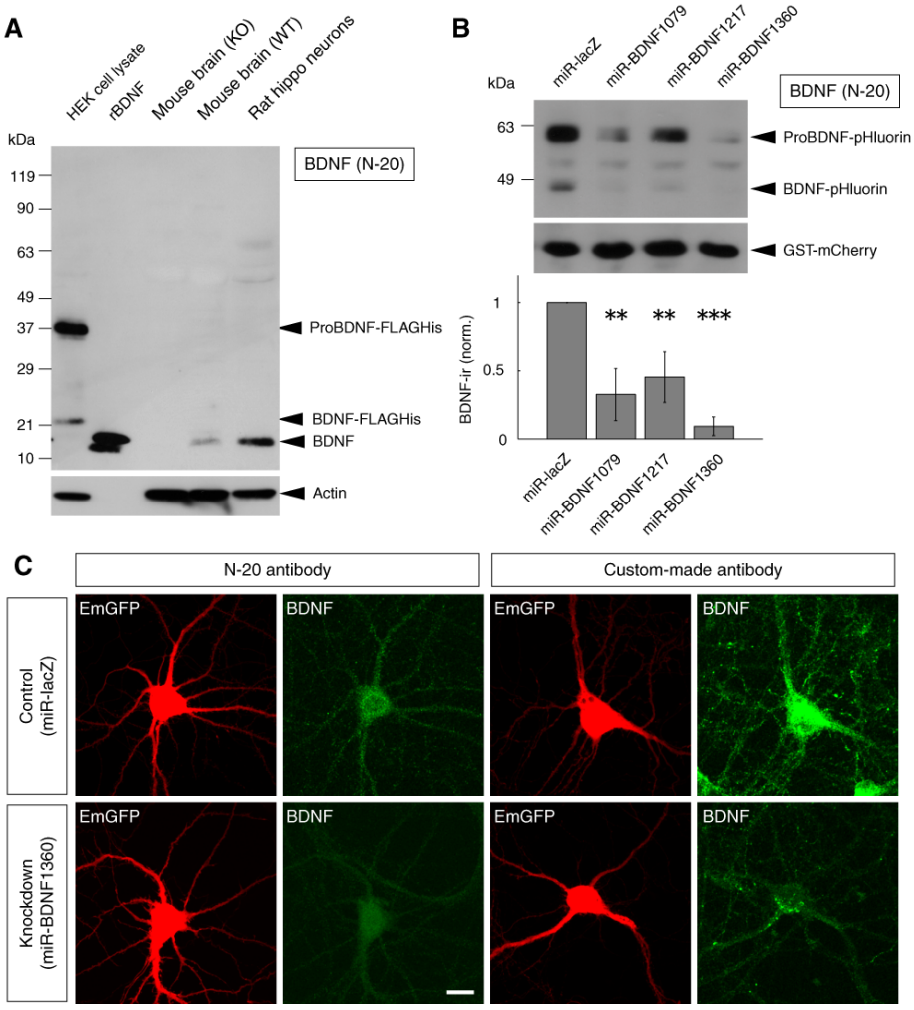
We have examined how BDNF-pHluorin was processed in COS-7 and cultured hippocampal neurons. Conditioned media and lysates of COS-7 cells expressing EGFP, BDNF-EGFP or BDNF-pHluorin were prepared, blotted and probed with the N-20 antibody. Both pro- and mature forms of BDNF were observed in the cell lysate, while secreted BDNF probes in the conditioned media were found as a converted, mature form (supplemental Fig. 4A).

We next immunoprecipitated secreted BDNF-pHluorin from the conditioned media of 28-DIV cultured hippocampal neurons using polyclonal antibody to GFP (custom-made), and prepared concentrated cell lysate of the neurons (supplemental Fig. 4B). When the blots

were probed with monoclonal anti-GFP antibody (MBL International), most BDNF-pHluorin in the conditioned media exhibited the mature form. In the cell lysate of the neurons, the blots reprobed with the N-20 antibody showed a faint proBDNF-pHluorin signal of an expected size (~60 kDa) besides ~55 kDa protein of unknown identity. Thus, it is likely that most BDNF-pHluorin had been converted to a mature form before it was secreted from the neurons, which exhibited spontaneous spiking activities. Non-secreted, transmembrane GluR1-pHluorin was detected only in the cell fraction, indicating that separation procedure between cells and media was carried out properly.

Supplemental Note 3: Stimulation with High Potassium Solution.

We have also stimulated cultured hippocampal neurons with a depolarizing extracellular solution with elevated K^+ concentration (50 mM) to examine if our findings were reproduced under a different stimulation condition. We switched from the normal extracellular solution to that with 50 mM Na^+ substituted with the equal amount of K^+ for stimulation, and then recorded from the neurons expressing BDNF-pHluorin, under the TIRF microscopy at a high spatiotemporal resolution. We observed that BDNF-pHluorin fluorescence at single vesicles changed transiently with various onset times after stimulation with a high K^+ depolarizing extracellular solution (supplemental Fig. 10A, C). The average traces of BDNF-pHluorin fluorescence with onset times aligned showed rise times and decay times similar to those examined with electrical stimulation (supplemental Fig. 10B, D). We found that the increases in BDNF-pHluorin fluorescence were spatially confined to the region of the fusion pore formed at the axon, whereas we observed rapid diffusion of BDNF-pHluorin around the fusion pore at the dendrite (supplemental Fig. 10E, F). Based on those findings, we concluded that differential exocytic fusion modes of BDNF-containing vesicles the axon and dendrite were due to intrinsic properties of neurons, regardless of the method for depolarizing the neurons.

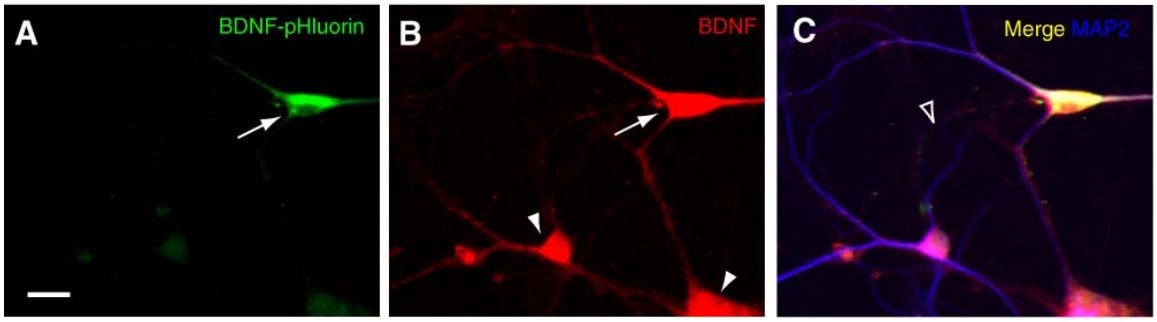


Supplemental Figure 1. Specificity of Antibodies to BDNF.

(A) Western blot analyses with N-20 antibody of wild-type and BDNF knockout mouse brain and rat cultured hippocampal neurons. Recombinant human BDNF (rBDNF) and the lysate of HEK cells expressing Flag- and His-tagged BDNF as positive controls. Actin was used as the loading control.

(B) The efficacy of BDNF knockdown vectors (miR-BDNF1079, miR-BDNF1217 and miR-BDNF1360) estimated by the BDNF expression level in HEK cells transfected with BDNF-pHluorin and the knockdown vectors, relative to that found in HEK293 cells transfected with the control knockdown vector (miR-lacZ). **, $p < 0.01$; ***, $p < 0.001$ compared with control (*t* test, $n = 5$ for each condition).

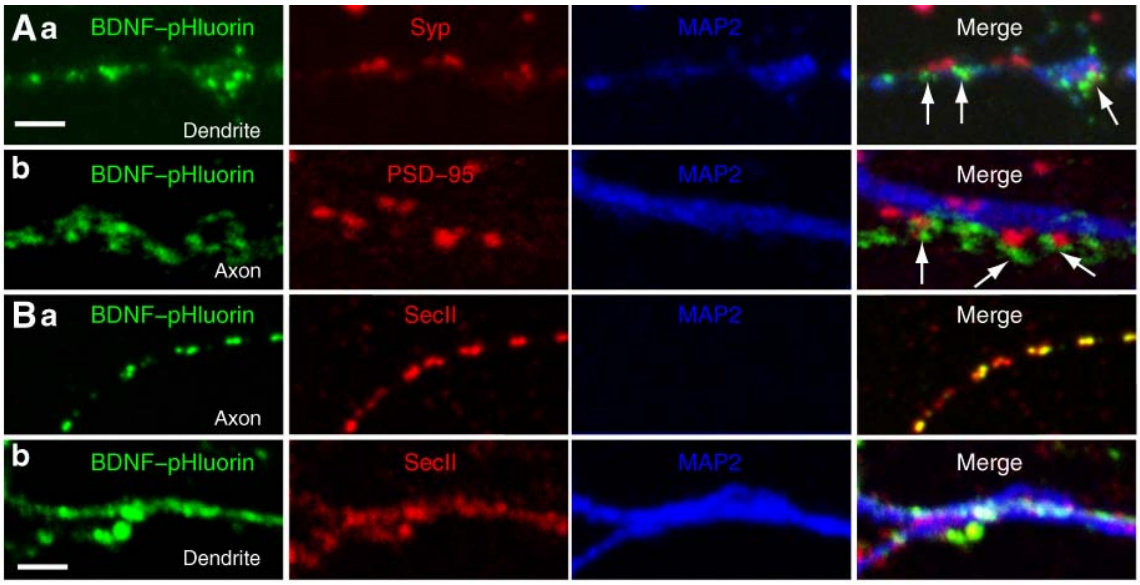
(C) Endogenous BDNF stained with two different polyclonal antibodies to BDNF in control and BDNF knockdown neurons. Scale bar, 20 μm.



Supplemental Figure 2. BDNF Expression Level in Neurons Transfected with BDNF-pHluorin.

(A, B) Images of example hippocampal neurons showing BDNF-pHluorin fluorescence (A) and BDNF immunoreactivity (B) in transfected (arrow) and un-transfected (arrowhead) neurons. Scale bar, 20 μm.

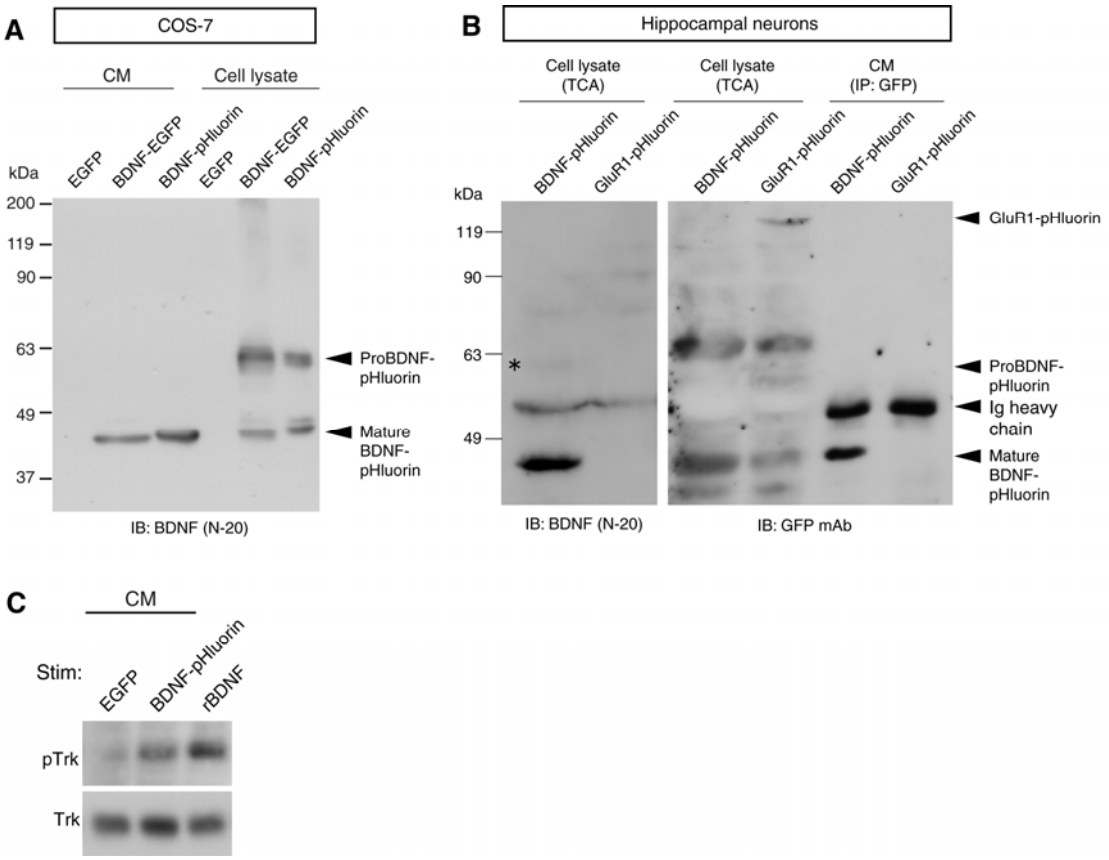
(C) Merge of pHluorin and BDNF immunofluorescence images shown in A & B, overlaid with immunostaining for MAP2 (blue). An open arrow indicates an axon.



Supplemental Figure 3. Co-localization of BDNF-pHluorin Puncta with Synaptic and Secretory Granule Markers.

(A) Synaptic and extrasynaptic localization of BDNF-pHluorin puncta in the axon (A_a) and dendrite (A_b), examined by triple immunostaining for EGFP (green), MAP2 (blue), and synaptic marker PSD-95 or synaptophysin (Syp). Arrows: BDNF-pHluorin puncta at the axon (A_a) and dendrite (A_b) that showing juxtaposition to the synaptic marker. Scale bar, 2 μm.

(B) Co-immunostaining for BDNF-pHluorin (green) and SecII (red), showing most BDNF-pHluorin puncta colocalized with SecII in axons (B_a) and dendrites (B_b). Scale bars, 2 μm.

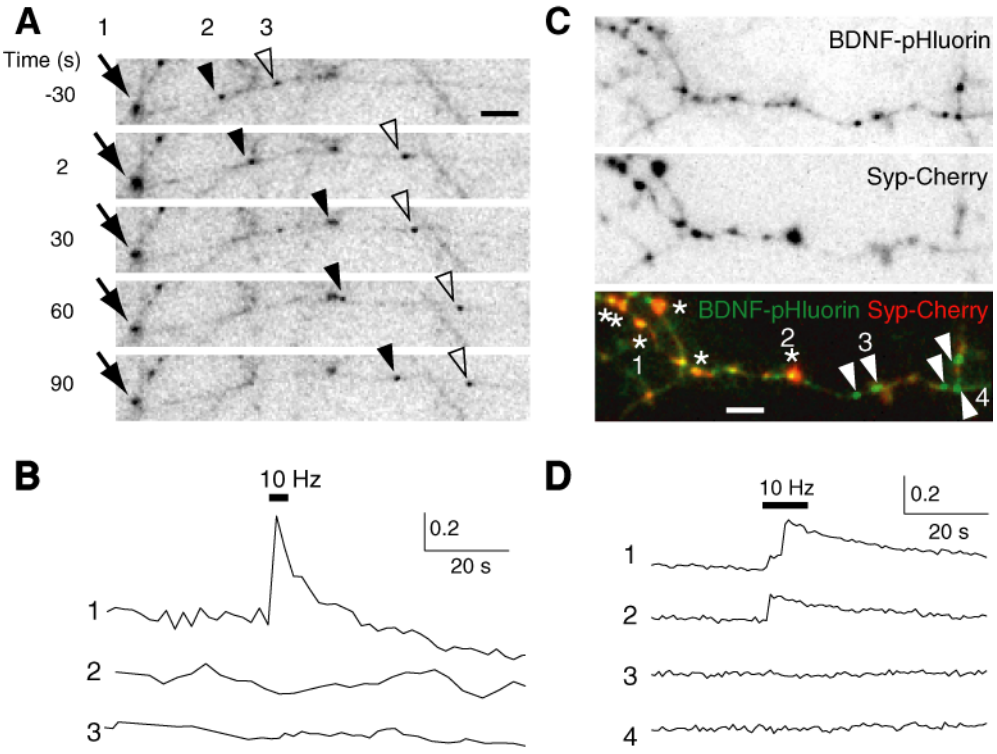


Supplemental Figure 4. Expression, Secretion and Biological Activity of BDNF-pHluorin.

(A) Western blot analysis of the conditioned media (CM) and lysate of COS-7 cells transfected with EGFP alone, BDNF-EGFP, or BDNF-pHluorin, with N-20 anti-BDNF antibody. Bands corresponding to BDNF probes with pro- and mature forms of BDNF (arrowheads).

(B) Western blots of cultured hippocampal neurons transfected with either BDNF-pHluorin or GluR1-pHluorin probed with anti-BDNF polyclonal (N-20) or anti-GFP monoclonal antibody. Cell lysates and CM were precipitated with trichloroacetic acid (TCA) and anti-GFP antibody, respectively. Asterisk, a weak band corresponding to proBDNF-pHluorin.

(C) Western blot analysis of the lysate of cultured cortical neurons, using antibodies to the total (Trk) or phosphorylated (pTrk) Trk receptors. The cortical neurons were treated as indicated above each lane. Conditioned media were collected from COS-7 cells transfected with EGFP alone or BDNF-pHluorin. "rBDNF", recombinant human BDNF.



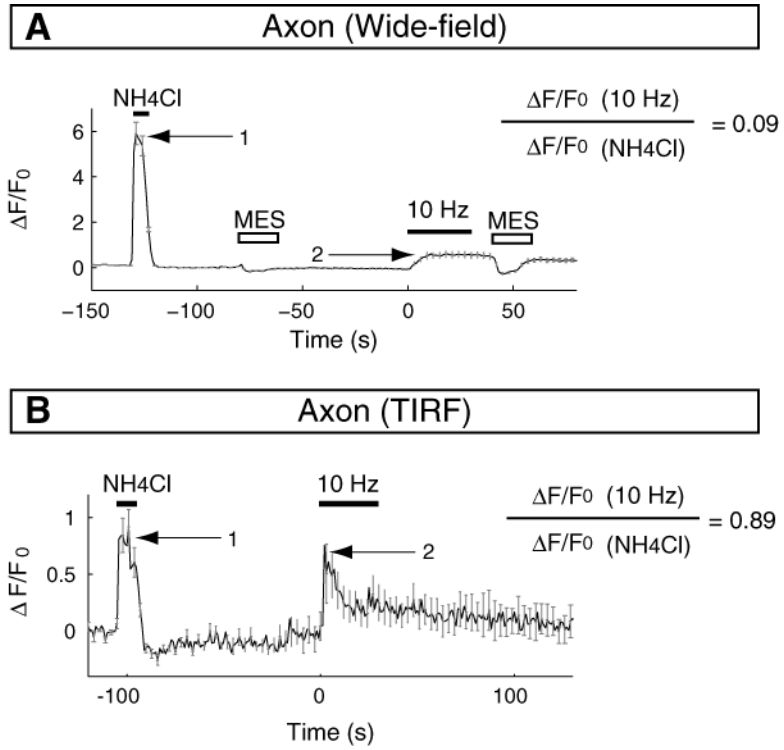
Supplemental Figure 5. Activity-Induced Fluorescence Transients Were Mainly Found at Immobile BDNF-pHluorin Puncta at Presynaptic Sites.

(A) Time-lapse images of an axon of a hippocampal neuron expressing BDNF-pHluorin. The onset time of stimulation was t = 0 s. Arrows and arrowheads mark immobile and mobile puncta, respectively.

(B) Fluorescence changes upon field stimulation (40 pulses, 10 Hz) at puncta marked in A (1: immobile; 2, 3: mobile).

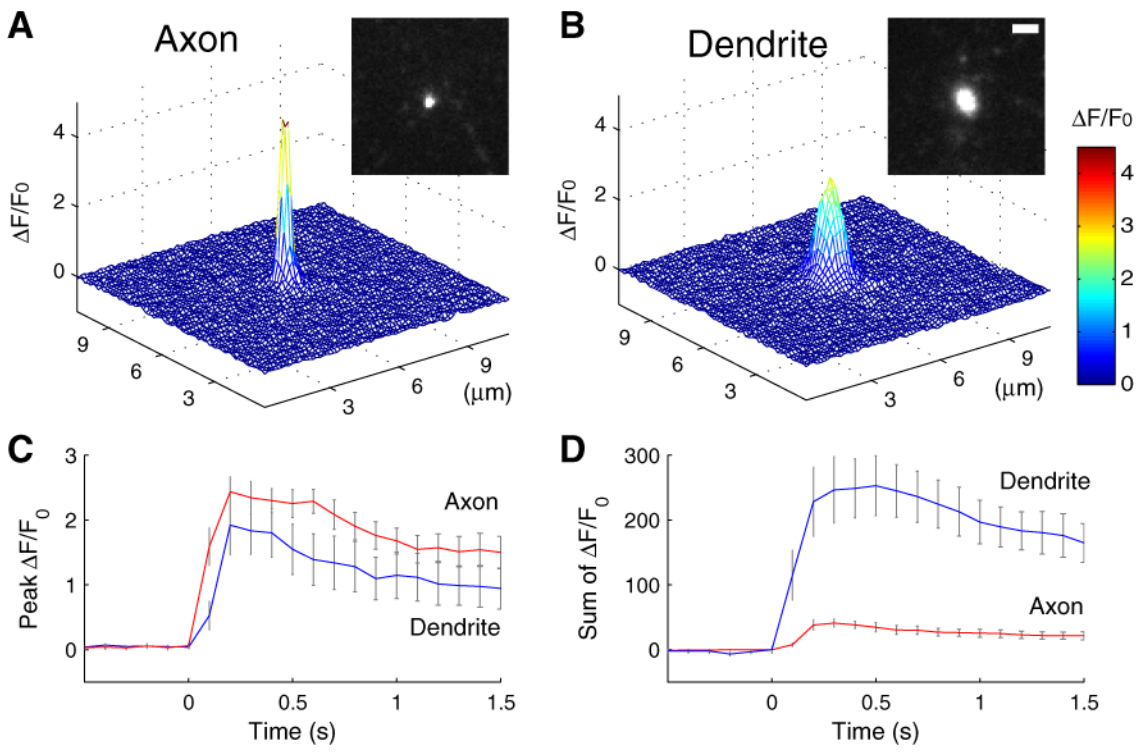
(C) Images of neurons co-transfected with BDNF-pHluorin (upper) and synaptophysin-cherry (Syp-Cherry; middle). Colored panel: overlaid images. **Asterisks**: puncta co-localized with synaptophysin puncta. **Arrowheads**: puncta not colocalized with synaptophysin.

(D) Fluorescence changes of BDNF-pHluorin puncta marked by numbers in C.



Supplemental Figure 6. Fraction of BDNF-pHluorin Vesicles Responding to Field Stimulation.

(A, B) Average traces of fluorescence changes at BDNF-pHluorin puncta in the axon, observed with either wide-field epi-fluorescence (A) or TIRF (B) microscopy. Peak $\Delta F/F_0$ (± SEM) values induced by NH_4Cl perfusion (pH = 7.4) and 10 Hz field stimulation were indicated arrows marked as 1 & 2, respectively.

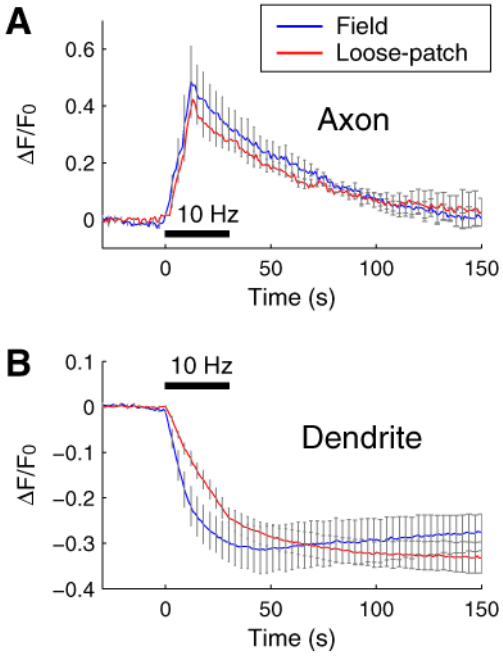


Supplemental Figure 7. Spatial Distribution of BDNF-pHluorin upon Fusion Pore Opening

(A, B) Representative images of BDNF-pHluorin spots at the time of their maximal intensities, and 3-D plots showing spatial distribution of $\Delta F/F_0$ at the spot. A color code used for the 3-D plots was shown on the right. Scale bar, 2 μm.

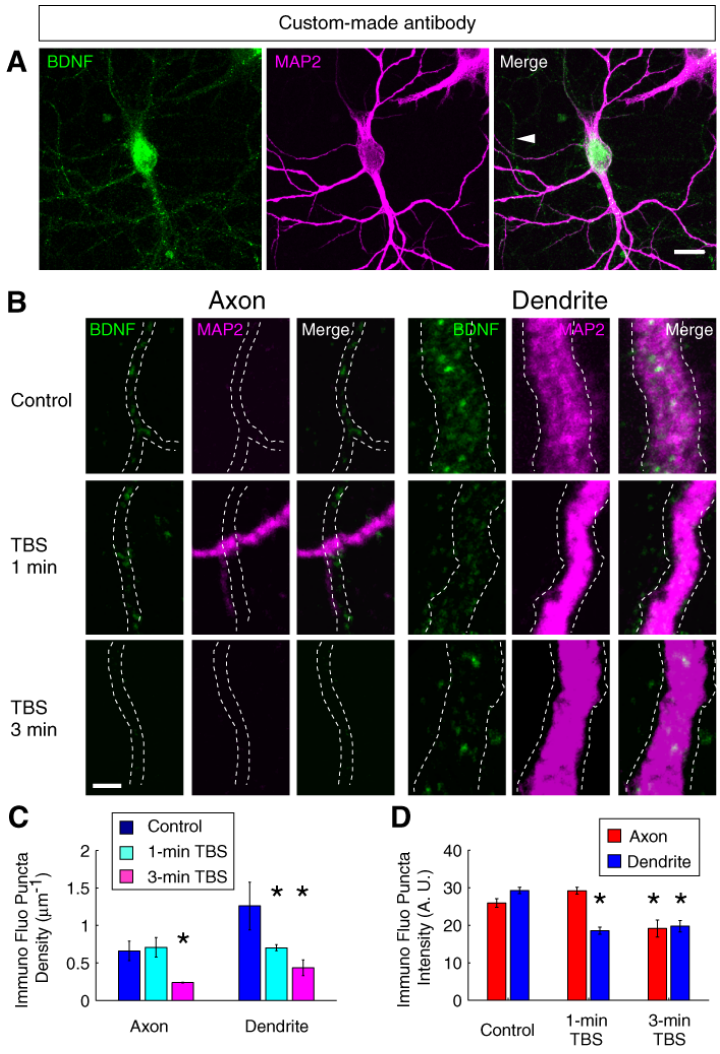
(C) Time course of the peak $\Delta F/F_0$ averaged over multiple vesicles (n = 8 for axons, n = 7 for dendrites). Error bars, SEM.

(D) Time course of the sum of $\Delta F/F_0$ averaged over multiple vesicles (n = 8 for axons, n = 7 for dendrites). The sum of $\Delta F/F_0$ reflects the fractional change of the total BDNF-pHluorin fluorescence in the evanescent field at a given time. Error bars, SEM.



Supplemental Figure 8. Efficacy of Field vs. Loose-Patch Stimulation in Triggering Vesicle Fusion.

Average changes (±SEM) of BDNF-pHluorin punctum fluorescence at axons (A) and dendrites (B), using either field or loose-patch stimulation of the same pattern (10 Hz, 300 pulses). Data from 89 and 153 puncta at axons and dendrites, respectively, were used for averaging.

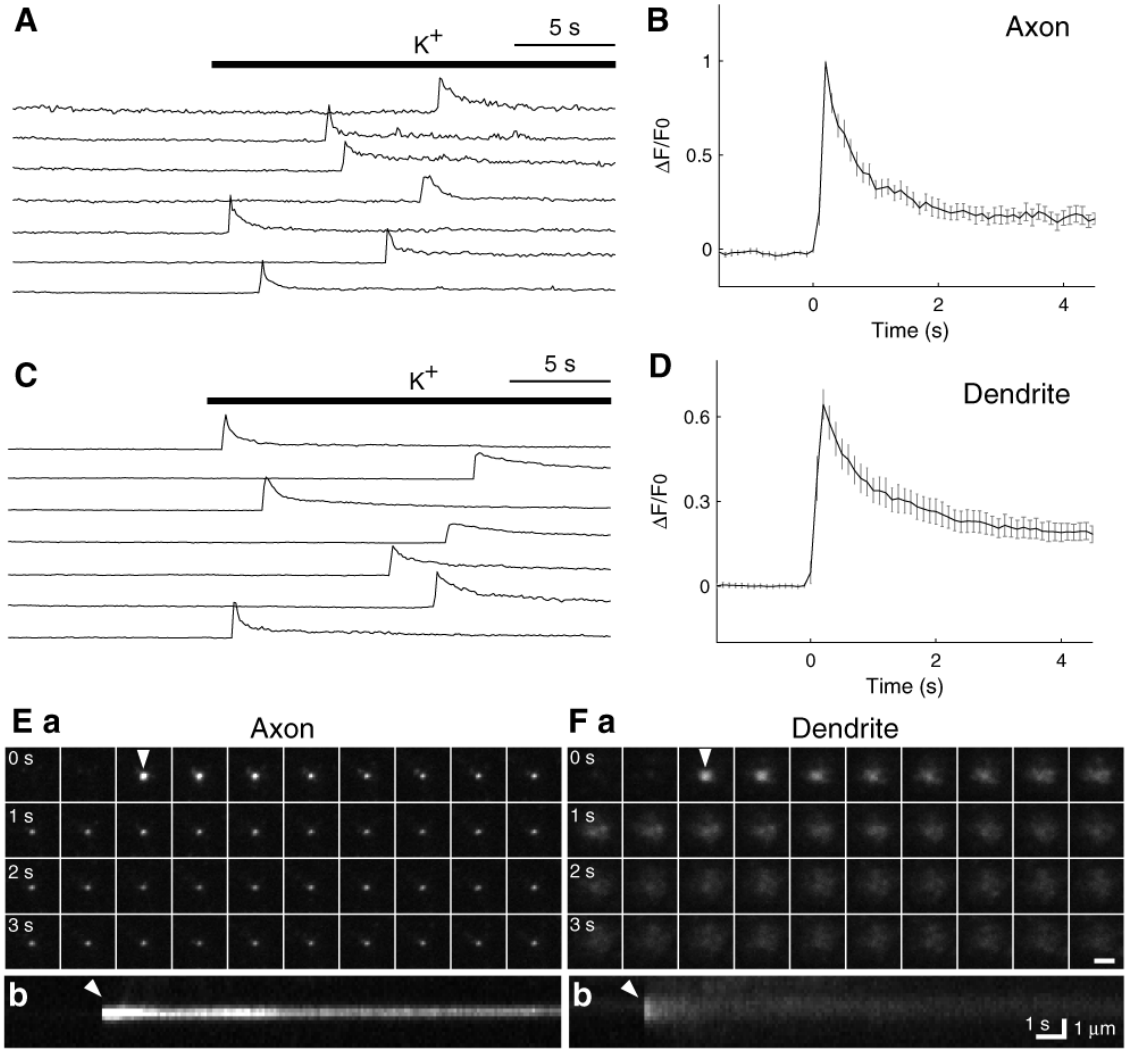


Supplemental Figure 9. Endogenous BDNF Examined with a Custom-made Antibody to BDNF.

(A) Endogenous BDNF at the axon and dendrite stained with antibodies to BDNF (gift from Dr. Ritsuko Katoh-Semba) and MAP2. Arrowhead, axon. Scale bar, 20 μm.

(B) Representative images of the axons and dendrites of neurons immunostained for the endogenous BDNF immediately after 1- or 3-min TBS. Imaging was performed with the consistent settings of the lasers and photomultipliers of a confocal microscope. Scale bar, 2 μm.

(C, D) Bar graphs showing the mean densities (C) and intensities (D) of immunofluorescent puncta at the axon and dendrite with or without to exposure to 1- or 3-min TBS for axons (n = 3 neurons for each condition). Error bars, SEM. *, $p < 0.05$.



Supplemental Figure 10. Stimulation with High K^+ Extracellular Solution.

(A, C) Sample traces of BDNF-pHluorin fluorescence changes (ΔF) measured at each vesicle at the axon (A) and dendrite (C) of neurons stimulated by perfusion (bar) with an extracellular solution containing 50 mM K^+ (bar).

(B, D) Average traces of BDNF-pHluorin fluorescence transients as those shown in A and C, normalized by the basal fluorescence (F_0) with the onset time of the transients aligned (n = 20 for axons, 32 for dendrites).

(E, F) Representative sequential images of single vesicles at the axon (E_a) and dendrite (F_a). Scale bar, 2 μm. Pseudo line scan images along the center of the spot for the vesicles (E_b, F_b). Arrowhead, opening of fusion pore.

Legend to supplemental Movie 1. Fusion of BDNF-pHluorin-containing Vesicles Induced by Electrical Stimulation at the Axon of Cultured Hippocampal Neuron.

A 25-s movie recorded with TIRF video microscopy (at frame rate of 10 Hz, shown in real time). The cultured neurons expressing BDNF-pHluorin were exposed to field stimulation (10 Hz, 1-ms pulse width, white trace) and the pHluorin fluorescence changes at three different puncta (marked by corresponding colored arrows) at the axon were shown in the color traces. The axon was identified with wide-field microscopy before the recording was switched to TIRF microscopy. Note that the pHluorin fluorescence spot remained similar size throughout the transient, indicating the absence of vesicle collapse following the opening of the fusion pore.

Legend to supplemental Movie 2. Fusion of BDNF-pHluorin-containing Vesicles Induced by Electrical Stimulation at the Dendrite of Cultured Hippocampal Neuron.

A 25-s movie recorded with TIRF video microscopy (at frame rate of 10 Hz, shown in real time). The cultured neurons expressing BDNF-pHluorin were exposed to field stimulation (10 Hz, 1-ms pulse width, white trace) and pHluorin fluorescence changes at three different puncta (marked by corresponding colored arrows) at the dendrite was shown in the color traces. The dendrite was identified with wide-field microscopy before the recording was switched to TIRF microscopy. Note the dilation of the pHluorin fluorescence spot and subsequent dispersion of pHluorin fluorescence, indicating full fusion of the vesicle and secretion of BDNF-pHluorin. The yellow arrow indicates dispersion of pHluorin fluorescence without a spot probably because the opening fusion pore was beyond the evanescent field.