BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin–β-catenin interactions

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Neurons of the vertebrate central nervous system have the capacity to modify synapse number, morphology, and efficacy in response to activity. Some of these functions can be attributed to activity-induced synthesis and secretion of the neurotrophin brain-derived neurotrophic factor (BDNF); however, the molecular mechanisms by which BDNF mediates these events are still not well understood. Using time-lapse confocal analysis, we show that BDNF mobilizes synaptic vesicles at existing synapses, resulting in small clusters of synaptic vesicles “splitting” away from synaptic sites. We demonstrate that BDNF’s ability to mobilize synaptic vesicle clusters depends on the dissociation of cadherin–β-catenin adhesion complexes that occurs after tyrosine phosphorylation of β-catenin. Artificially maintaining cadherin–β-catenin complexes in the presence of BDNF abolishes the BDNF-mediated enhancement of synaptic vesicle mobility, as well as the longer-term BDNF-mediated increase in synapse number. Together, this data demonstrates that the disruption of cadherin–β-catenin complexes is an important molecular event through which BDNF increases synapse density in cultured hippocampal neurons.

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Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; DIV, day in vitro; LTP, long-term potentiation; SV, synaptic vesicle.

The online version of this article contains supplemental material.
Results

SVs are the morphological hallmark of all chemical synapses, and are recruited to points of cell–cell contact during presynaptic development. Two pools of SVs have previously been identified by time-lapse analysis; a relatively stable pool that remains stationary for hours and localizes primarily at presynaptic boutons, and a mobile pool that translocates along the axon (Ahmari et al., 2000; Krueger et al., 2003). Stable vesicle clusters are thought to form when mobile packets of SVs become “trapped” at nascent synapses. This process is now believed to depend on signals that are activated after the interaction of cell adhesion molecules (Scheiffele et al., 2000; Biederer et al., 2002; Bamji et al., 2003).

BDNF transiently disrupts synaptic vesicle localization by dissolving cadherin-β-catenin complexes

To study the real-time effects of BDNF on SV dynamics, hippocampal neurons were transfected with the integral SV protein synaptophysin fused to GFP (synaptophysin-GFP) and imaged using time-lapse microscopy. It has previously been demonstrated that the pattern of synaptophysin-GFP expression is similar to that of endogenous synaptic vesicle proteins (Bamji et al., 2003), and that these fusion proteins do not compromise the secretory physiology of the synapse (Sankaranarayanan and Ryan, 2000). Synaptophysin-GFP is known to label both the mobile and stable pools of SVs. Two studies detailing the dynamics of SV clusters have concluded that stationary puncta typically represent synaptic sites. Consequently, to look at the dynamics of SVs at extant synapses, we focused on the SV clusters that were stably localized along the axon for the duration of our imaging (Ahmari et al., 2000; Krueger et al., 2003).

In control cultures, fluctuations in synapse size (measured as the length of the major axis of each synaptophysin-GFP puncta) were minimal (Fig. 1, A–C and J; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200601087/DC1). After acute treatment with BDNF, however, we observed a transient diffusion of SVs along the axon and into perisynaptic regions (Fig. 1, D–I and J). BDNF was added at time point 0, and left in the medium for the duration of imaging. Although the time of maximal diffusion varied from synapse to synapse, on average there was a 1.6-fold increase in the length of the major axis of stable synaptophysin-GFP puncta after BDNF treatment, both at 7 DIV, when synapses are beginning to form, and at 12 DIV, when synapses are more mature (Fig. 1 J).

To determine whether the elongated pattern of synaptophysin-GFP expression is caused by diffusion along the axon surface after depolarization-stimulated exocytosis, which has been previously observed (Tanaka et al., 2000), cells were preincubated for 16 h with 10 nM tetanus toxin to block exocytosis. Tetanus toxin did not affect the dispersal of SVs after BDNF treatment, indicating that the elongated pattern of synaptophysin-GFP expression is caused by dispersal of SVs within the axon (Fig. 2 A).

We have recently shown that inhibiting cadherin–β-catenin interactions results in a diffuse pattern of synaptophysin-GFP localization along the axon (Bamji et al., 2003). Therefore, we hypothesized that BDNF may regulate vesicle clustering by disrupting cadherin–β-catenin complexes. Previous reports have shown that phosphorylation of β-catenin on tyrosine 654 by Src family kinases greatly decreases the affinity of β-catenin for cadherins (Roura et al., 1999; Piedra et al., 2001). Interestingly, we observed an increase in β-catenin tyrosine phosphorylation and a decrease in cadherin–β-catenin interactions 10 min after BDNF treatment (Fig. 1 K). β-catenin phosphoryrosine levels diminished and cadherin–β-catenin interactions were largely restored 30 min after BDNF treatment (Fig. 1 K).
We have also shown the disruption of cadherin–β-catenin interactions by coimmunoprecipitation using a pan-cadherin antibody (unpublished data). Our results indicate that BDNF can transiently disrupt cadherin–β-catenin complexes with a time course similar to that of BDNF-induced SV diffusion. This experiment does not show that the synaptic pool of cadherin is specifically affected; however, it is well documented that TrkB is expressed at synapses (Drake et al., 1999) and that the majority of cadherin and β-catenin are localized at cell contacts, most of which form synapses in hippocampal cultures (Fannon and Colman, 1996; Benson and Tanaka, 1998; Tang et al., 1998; Bozdagi et al., 2000, 2004; Tanaka et al., 2000; Togashi et al., 2002).

To further examine the role of the cadherin–β-catenin adhesion complex in BDNF-mediated vesicle mobilization, we inhibited the BDNF-mediated dissociation of cadherin–β-catenin complexes by expressing a β-catenin point mutant that cannot be phosphorylated on tyrosine residue 654 (β-catenin Y654F). The β-catenin Y654F mutation has previously been shown to prevent Src-dependent dissociation of the cadherin–β-catenin complex (Murase et al., 2002; Lilien and Balsamo, 2005). Expression of β-catenin Y654F completely abolished the transient diffusion of vesicles seen after BDNF application, indicating that the disruption of cadherin–β-catenin complexes is required to observe significant SV dispersion (Fig. 2 C). In contrast, overexpression of wild-type β-catenin did not significantly inhibit SV dispersion (Fig. 2 B). These results indicate that BDNF disrupts synaptically localized, stable SV clusters by disrupting cadherin–β-catenin complexes, which are important for localizing SVs to specified regions underlying active zones.

We have previously shown that synaptophysin-GFP clusters are more dynamic in cells lacking β-catenin (Bamji et al., 2003). Cells lacking β-catenin were generated by culturing neurons from β-catenin fl ox mice (Huelsken et al., 2001) and transfecting them with a vector expressing the Cre recombinase. Changes in the length of four representative synaptophysin-GFP positive puncta in four different experimental conditions are depicted in Fig. 2 (E–G). Analysis of the length of the major axis of individual synaptophysin-GFP puncta revealed
Hippocampal neurons were cultured from seven of a total 15 mice. To test the idea that BDNF-mediated vesicle dispersal is due to increased BDNF-promoted SVs, we measured the percentage of change in the length of the major axis of four representative synaptophysin-GFP puncta versus time (Fig. 2A). Treatment with tetanus toxin did not affect the dispersal of SVs. In the presence of BDNF, there is a transient diffusion of SVs into perisynaptic regions, as indicated by an increase in the length of synaptophysin-GFP fluorescence along the axon (Fig. 2B). Phosphorylation of β-catenin at Y654 increased the dynamics of SVs, with a transient diffusion of SVs into perisynaptic regions, as indicated by an increase in the length of synaptophysin-GFP fluorescence along the axon (Fig. 2C). The addition of BDNF to hippocampal neurons lacking β-catenin did not detectably increase the instability in SV localization beyond the increase resulting from the absence of β-catenin alone (Fig. 2D and F). This is consistent with the possibility that BDNF’s effects are mediated by dissolution of cadherin–β-catenin interactions.

**BDNF-treatment enhances the splitting of synaptic vesicle clusters**

Compared with control cultures, which displayed minimal SV fluctuations, BDNF results in a 1.7-fold increase in the average frequency of SV cluster splitting over a 20-min observation period (Fig. 3C). The increase in splitting frequency was observed throughout the 20-min observation period (Fig. 3B, arrow at 14 min, and Video 2). The persistence of SV splitting events suggested that there might be a long-term destabilization of presynaptic compartments. To explore this, we also quantified the frequency of splitting after 3 d of continuous BDNF treatment. Although the overall frequency of vesicle splitting was slightly lower in the older control cultures, BDNF still enhanced the frequency of SV cluster splitting (Fig. 3C). Interestingly, after 3 d of BDNF treatment, the size of the clusters was relatively stable over time (D). After treatment of β-catenin–expressing neurons with BDNF, there is a transient diffusion of SVs into perisynaptic regions as indicated by an increase in the length of synaptophysin-GFP fluorescence along the axon (E). Expression of Cre recombinase to ablate β-catenin, results in increased dynamics of SVs, as indicated by dramatic fluctuations in synaptophysin-GFP puncta length along the axon (F). Addition of BDNF to cells lacking β-catenin does not further increase the dynamics of SV cluster length (G) compared with the fluctuations observed in cells not treated with BDNF, but also lacking β-catenin (F).
synaptophysin-GFP clusters was similar in treated compared with untreated cultures (unpublished data). Among other possibilities, this suggests that the content of individual SV clusters may be replenished by the coalescence of mobile vesicle clusters with stable clusters, and possibly also by enhanced SV synthesis. Consistent with the latter possibility, BDNF has been shown to enhance the synthesis of some SV components (Tartaglia et al., 2001).

When cadherin–β-catenin complexes were maintained by expression of the β-catenin Y654F point mutant, the BDNF-mediated increase in the splitting of vesicle clusters was abolished (Fig. 3 C). This suggests that BDNF regulates the splitting of SV clusters, in part, through regulation of cadherin–β-catenin association. Cells expressing wild-type β-catenin did respond to BDNF with increased SV cluster splitting, but the magnitude of this effect was somewhat diminished compared with that observed in untransfected cells (Fig. 3 C). This intermediate effect was seen throughout the study, and it is most likely caused by an enhanced probability of cadherin–β-catenin association in cells overexpressing wild-type β-catenin. The absence of a more dramatic result probably reflects the fact that β-catenin that is not associated with cadherin is highly unstable.

Maintenance of cadherin–β-catenin interactions prevents BDNF-mediated synapse formation

BDNF signaling through its receptor TrkB has been reported to increase synapse formation, as well as neurite branching, in hippocampal neurons (Vicario-Abejon et al., 1998; Collin et al., 2001; Danzer et al., 2002; Tolwani et al., 2002; Tyler and Pozzo-Miller, 2001, 2003). Using two approaches, we observed that the addition of 100 ng/ml BDNF for 3 d mediates an obvious increase in the density of SV clusters, and that this event is dependent on BDNF’s modulation of cadherin–β-catenin interactions. Compared with untreated cultures (Fig. 4, A and I), the addition of BDNF to hippocampal cultures transfected with synaptophysin-GFP resulted in an increased density of synaptophysin-GFP puncta (Fig. 4, B and I). The BDNF-mediated increase in SV puncta density was prevented by coexpression of β-catenin Y654F (Fig. 4, C and I), but not wild-type β-catenin (Fig. 4, C and I).

The density of endogenous synaptophysin puncta was also examined. Compared with untreated cultures (Fig. 4 E, red), there was an overall increase in the number of endogenous synaptophysin-immunopositive puncta in cultures treated with 100 ng/ml BDNF for three days (Fig. 4, F–H, red). This is consistent with published results and could be caused, in part, by an increase in axonal extension and branching (Vicario-Abejon et al., 1998; Collin et al., 2001; Danzer et al., 2002; Tolwani et al., 2002; Tyler and Pozzo-Miller, 2001, 2003). To more specifically examine the density of SV clusters, synaptophysin-immunopositive puncta were counted within GFP-labeled processes (Fig. 4, E–H [yellow puncta], and J). Treatment with BDNF of wild-type neurons, or of neurons overexpressing full-length β-catenin, increased the density of synaptophysin-immunopositive puncta (Fig. 4, F, G [yellow puncta], and J). In contrast, expression of β-catenin Y654F completely inhibited
BDNF’s effects on the density of synaptophysin-immunopositive puncta (Fig. 4, H and I). It is important to note the internal control in these cultures. The overall density of synaptophysin-immunopositive puncta was increased in cultures treated with BDNF (compare red boutons in Fig. 4 E to those in F–H). However, the number of synaptophysin-immunopositive puncta in individually labeled β-catenin Y654F-expressing cells was similar to controls (Fig. 4, E and H).

SV clusters are expected to include both the mobile pool of vesicles and the pool of vesicles stably localized at synapses. To identify the pools affected by BDNF, we imaged hippocampal neurons transfected with synaptophysin. As expected, there was a twofold increase in the total number of SV clusters after BDNF treatment for 3 d. Moreover, BDNF increased the density of both the stable and mobile SV clusters (Fig. 4 K). Neurons expressing β-catenin Y654F did not exhibit any
significant increase in the density of total, stable, or mobile vesicle clusters compared with untreated cells.

To determine if the stably localized SV clusters represented synaptic sites, we immunostained control and BDNF-treated cultures with an antibody specific for the excitatory postsynaptic marker PSD-95. The density of SV clusters apposed to PSD-95 (Fig. 5 E) was similar to the density of stable SV clusters (Fig. 4 K). As expected, treatment of neurons with BDNF for 3 d resulted in an overall increase in the density of synaptophysin-GFP–positive puncta compared with controls (compare Fig. 5, A and C). The increase in the number of PSD-95–positive puncta that colocalized with synaptophysin-GFP (Fig. 5 E) was very similar to the increase in the number of stable SV clusters (Fig. 4 K). Interestingly, BDNF also increased quite dramatically the density of synaptophysin-GFP–positive puncta that did not colocalize with PSD-95 puncta (Fig. 5 F). These may represent synaptophysin-GFP–positive puncta at inhibitory synapses, mobile SV clusters or both. The BDNF-promoted increases in synaptophysin-GFP puncta apposed and not apposed to PSD-95 were each blocked by expression of β-catenin Y654F.

**Discussion**

Understanding the molecular events that control synapse formation and modification is an important objective in neuroscience. BDNF is synthesized and secreted in response to neuronal activity, and it has been shown to enhance synaptic efficacy and promote the formation of new synapses both in vitro and in vivo (Bramham and Messaoudi, 2005). Despite the great interest in understanding how secreted factors regulate synapse form and function, the mechanisms underlying BDNF’s actions are still not well understood. In this study, we have shown that BDNF induces the morphological rearrangement of individual presynaptic compartments and promotes the formation of new synapses, in part, by disrupting cadherin–β-catenin complexes. Specifically, we have shown that (a) BDNF signaling reduces the interaction between cadherins and β-catenin; (b) BDNF...
treatment results in an acute dispersion of SVs, which is inhibited by artificially maintaining cadherin–β-catenin interactions by expressing a β-catenin mutant unable to be phosphorylated at Y654; and (c) BDNF enhances the formation of new synapses, which is inhibited by artificially maintaining cadherin–β-catenin interactions. A model illustrating our observations is presented in Fig. 6. As both the “splitting” of synaptically localized SV clusters and the increase in synapse density can be inhibited by inhibiting the dissociation of cadherin–β-catenin interactions, we have speculated that SV cluster splitting contributes to the formation of new synapses. This model has yet to be rigorously tested in vivo.

**BDNF and cadherin-β-catenin complexes enhance the motility of SV clusters**

We have previously demonstrated that cadherin–β-catenin complexes and F-actin organization.

Figure 6. **Model for the role of cadherin-β-catenin interactions in BDNF-mediated presynaptic plasticity.** (1) In the absence of TrkB-mediated signaling, β-catenin is not phosphorylated at Y654 and is associated with cadherins at the synapse, providing a scaffold through the β-catenin PDZ domain interaction motif for recruitment of scaffold proteins and synaptic vesicle to the synapse. (2) Activation of TrkB receptor tyrosine kinase by BDNF results in the phosphorylation of β-catenin on Y654. This causes the dissociation of β-catenin from cadherins, and disruption of the signals responsible for localizing SVs to the presynaptic compartment. (3) Subsequently, SVs disperse along the axon into perisynaptic regions. (4) β-catenin dephosphorylation and reassociation with cadherin may occur after the internalization of TrkB that can occur within 5 min of BDNF treatment. As a result, SVs recluster at synaptic zones; however, presynaptic compartments are altered and there is a persistent increase in the rate of small SV clusters splitting away from the SV cluster at the active zone. We hypothesize that the increased rate of SV cluster splitting may lead to an increase in the number of mobile SV clusters, and help to promote an increase in the overall density of synapses along the axon as well.

**BDNF and cadherin-β-catenin complexes enhance synapse formation**

Although the exact mechanisms underlying BDNF-induced synapse formation are unclear, it is possible that BDNF promotes the formation of new synapses, in part, by increasing the density of mobile SV clusters, which are subsequently localized at new points of contact. The BDNF-mediated increase in mobile vesicle cluster density requires the dissociation of cadherin–β-catenin clusters and may be caused, in part, by the splitting of preexisting SV clusters. Transcriptional up-regulation of SV-associated proteins may also be involved, as both BDNF and β-catenin can
regulate transcriptional events. Studies using time-lapse imaging have revealed that the overwhelming majority of contacts made between dendritic filopodia and axons are not stabilized, but are retracted within minutes of their formation (Jontes et al., 2000). The increased number of mobile vesicle clusters may increase the probability of contact stabilization and, ultimately, the formation of new synapses. Indeed, many of these mobile SV clusters are exocytosis competent (Krueger et al., 2003), and filopodia are known to be stabilized in the presence of local calcium transients (Lohmann et al., 2005).

It has recently been demonstrated using conditional TrkB-null mice that BDNF and TrkB signal in a cell-autonomous manner to promote excitatory synapse formation in the hippocampus (Luikart et al., 2005). Specific deletion of TrkB from presynaptic CA3 pyramidal neurons resulted in a decreased number of presynaptic terminals, with multiple postsynaptic compartments forming contacts with individual boutons (Luikart et al., 2005). Although controversial, it has been suggested that one mode of synapse formation is the splitting of individual synapses into two synapses, with perforated synapses being an intermediary (Nikonenko et al., 2002). In these mice, deletion of presynaptic TrkB may have prevented the mobilization of synaptic components such as SVs and thereby prevented the formation of new presynaptic boutons. It will be interesting to see whether artificial maintenance of cadherin–β-catenin clusters in vivo results in a phenotype similar to that seen after TrkB deletion. BDNF has also been shown to promote the formation of inhibitory, GABAergic synapses in vivo, as well as in vitro (Vicario-Abejon et al., 1998; Collin et al., 2001; Rico et al., 2002).

Interestingly, previous reports have shown that decreasing cell–cell adhesion increases the formation of new synapses. For example, the treatment of Aplysia californica sensory neurons with serotonin (5-HT) enhances the formation of new synaptic varicosities by down-regulating the Aplysia californica cell adhesion molecule (Han et al., 2004). Similarly, a decrease in the Drosophila melanogaster cell adhesion molecule fasciclin II is necessary for synapse sprouting at the neuromuscular junction in response to increased neuronal activity and cAMP concentration (Schuster et al., 1996a,b). Our demonstration that the dissolution of cadherin–β-catenin adhesive complexes at synapses promotes synaptic remodeling in response to BDNF further supports the hypothesis that synapse formation and plasticity are regulated, in part, by control of cell adhesion molecule function.

Materials and methods

Neuronal cultures

Rat hippocampi from E18 fetal rats were prepared as previously described (Xie et al., 2000) and plated at a density of 130 cells/mm². For time-lapse studies, neurons were transfected using Effectene (QIAGEN) transfection reagents and plated at a density of 130 cells/mm². For time-lapse imaging, neurons were transfected using Effectene (QIAGEN) transfection reagents and plated at a density of 130 cells/mm². For time-lapse imaging, neurons were transfected using Effectene (QIAGEN) transfection reagents and plated at a density of 130 cells/mm².

Immunoblot analysis and immunohistochemistry

Protein extracts were prepared from 12 DIV primary hippocampal cultures treated with either media alone or 100 ng/ml BDNF for 10 or 30 min. Extracts were immunoprecipitated with monoclonal anti-β-catenin (Zymed Laboratories) and immunoblots were probed with anti-phosphotyrosine (4G10; Cell Signaling Solutions) and anti-N-cadherin (a gift from D. Colman, McGill University, Montreal, Quebec, Canada). Proteins were visualized using enhanced chemiluminescence. Exposed film was scanned and the brightness and contrast of entire images was moderately adjusted using Photoshop (Adobe) after recommended, scientifically acceptable procedures, and no information was obscured or eliminated from the original (Rossner and Yamada, 2004).

For immunohistochemistry, neuronal cultures were fixed in 4% paraformaldehyde/4% sucrose for 10 min, permeabilized in 0.1% Triton X-100 for 10 min, and then blocked in 10% goat serum for 1 h at room temperature. Primary antibodies were applied in 1% goat serum overnight at 4°C, and secondary antibodies were applied in 1% goat serum for 1 h at room temperature. The primary antibodies used were mouse anti-synaptophysin (Sigma-Aldrich) and mouse anti-PSD-95 (Affinity BioReagents, Inc.). The secondary antibodies used were Alexa Fluor 488 and Texas red– conjugated goat anti-mouse or goat anti–rabbit IgGs (Invitrogen). n = at least 10 neurons per condition from at least three separate cultures.

Image analysis and quantification

Dispersion of SVs along the axon. Neurons transfected with synaptophysin-GFP were imaged every minute using a microscope (LSM 5 Pascal; Carl Zeiss Microlmaging, Inc.; 63×, 1.4 NA, oil Plan-Apochromat objective) and the corresponding LSM 5 Pascal Software. All images were captured with the same exposure time. Quantification of major axis length for each punctum over time was analyzed using LSM 5 Pascal Software. In brief, a short line was drawn along the axon and through the major axis of the puncta and analyzed at time point 0. The profile of the fluorescence intensity along the fixed line was then obtained for each time point using the “profile” function. For the analysis of each puncta, the length of the major axis at each time point was calculated with the length of the major axis at time point 0. All puncta that remained localized in the same spot along the axon during the 20–30 min imaging period were quantified. t tests were used for all statistical analyses.

Density of synaptophysin-GFP puncta. To quantify the density of synaptophysin-GFP puncta per axon length, images were imported into Image (National Institutes of Health), where puncta were identified and analyzed at a threshold of 55 and a minimum pixel size of 10. At least a 2,500-μm axon length from 10–30 cells from at least three separate cultures was analyzed per condition. To determine the density of synaptophysin and PSD-95 clusters, immuno-positive puncta along at least 1,500 μm of GFP-labeled axon length from 10–30 cells from at least three separate cultures were counted per condition. In brief, neurons were transfected with GFP or synaptophysin-GFP at 10 DIV and treated with BDNF at 11 DIV. 3 d later, cells were fixed and immunostained for synaptophysin or PSD-95, respectively. All images were captured on the LSM 5 Pascal scanning confocal microscope using constant settings. GFP-labeled axons were then measured, and all visible synaptophysin-positive puncta that colocalized with GFP-labeled axons were counted. t tests were used for all statistical analyses.

Online supplemental material

Video 1 shows that there is minimal variability in stably localized synaptophysin-GFP puncta over time in untreated cells. Video 2 shows that BDNF induces SV dispersal and increased splitting of SV clusters from stably localized synaptophysin-GFP-labeled puncta. Video 3 shows that BDNF induces SV diffusion and the splitting of SV clusters. Online supplemental material is available at http://jcb.org/cgi/content/full/jcb.200601087/DC1.

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