BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons

Sandra Dieni,1 Tomoya Matsumoto,2 Martijn Dekkers,2 Stefanie Rauskolb,2 Mihai S. Ionescu,2 Ruben Deogracias,2 Eckart D. Gundelfinger,3 Masami Kojima,4,5 Sigrun Nestel,1 Michael Frotscher,1 and Yves-Alain Barde2

1Department of Neuroanatomy, Institute of Anatomy and Cell Biology, Albert-Ludwigs-University Freiburg, 79104 Freiburg, Germany
2Biozentrum, University of Basel, CH-4056 Basel, Switzerland
3Department of Neurochemistry and Molecular Biology, Leibniz Institute for Neurobiology, 39118 Magdeburg, Germany
4Biointerface Research Group, Health Research Institute, National Institute of Advanced Industrial Science and Technology, Ikeda 563-8577, Japan
5Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

Although brain-derived neurotrophic factor (BDNF) regulates numerous and complex biological processes including memory retention, its extremely low levels in the mature central nervous system have greatly complicated attempts to reliably localize it. Using rigorous specificity controls, we found that antibodies reacting either with BDNF or its pro-peptide both stained large dense core vesicles in excitatory presynaptic terminals of the adult mouse hippocampus. Both moieties were ~10-fold more abundant than pro-BDNF. The lack of postsynaptic localization was confirmed in Bassoon mutants, a seizure-prone mouse line exhibiting markedly elevated levels of BDNF. These findings challenge previous conclusions based on work with cultured neurons, which suggested activity-dependent dendritic synthesis and release of BDNF. They instead provide an ultrastructural basis for an anterograde mode of action of BDNF, contrasting with the long-established retrograde model derived from experiments with nerve growth factor in the peripheral nervous system.

Introduction

Polarized cells use well-conserved mechanisms to sort proteins into specific compartments (Mellman and Nelson, 2008), providing them with a directionality that is critically important for both their function and meaningful integration into tissues. Central nervous system (CNS) neurons are prototypically polarized cells with specialized axonal and dendritic compartments that play essential roles in intercellular signaling. Although neurons typically communicate by releasing low–molecular weight neurotransmitters accumulated in synaptic vesicles, they also store and release peptides or small proteins such as brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family. BDNF is known to regulate a wide variety of brain functions in humans, ranging from food intake (Gray et al., 2006) to memory retention (Egan et al., 2003). Indeed, a single amino acid replacement in pro-BDNF has been convincingly shown to correlate with a diminished capacity to remember words and sentences (Egan et al., 2003; Cathomas et al., 2010). Furthermore, in animal models of disease, reduced levels of BDNF have been associated with several conditions, including depression (Calabrese et al., 2007), Rett syndrome (Chang et al., 2006), and Huntington’s disease (Zuccato et al., 2010).

Although many aspects of BDNF biology in the adult brain are thus beginning to be well appreciated, the subcellular localization of this secreted protein in neurons of the adult CNS is still very unclear, in large part because of the very low levels of endogenous BDNF. To address this question, several studies have resorted to neuronal cultures prepared from the embryonic
rodent hippocampus (Goodman et al., 1996; Hartmann et al., 2001; Kojima et al., 2001; Egan et al., 2003; Adachi et al., 2005; Dean et al., 2009; Matsuda et al., 2009; Jakawich et al., 2010). In most cases, conclusions about the localization of BDNF were inferred from visualization experiments using transfected tagged BDNF constructs, with recent studies concluding that BDNF is transported in and released from both axons and dendrites (Adachi et al., 2005; Dean et al., 2009; Matsuda et al., 2009; Jakawich et al., 2010). As firmly established by in situ hybridization studies, the Bdnf gene is expressed in an activity-dependent fashion by numerous excitatory neurons (Zafra et al., 1990), with protein levels increasing by ~10-fold during the first 3 wk after birth, in parallel with the development of synaptic activity (Tao et al., 1998; Kolbeck et al., 1999). Nonetheless, BDNF remains, even in the adult brain, an extremely rare protein, making its unambiguous detection in vivo a challenging task that is further complicated by the early death of Bdnf-null mutant animals, thus precluding their use as age-matched controls (CONs; Ernfors et al., 1994; Jones et al., 1994).

Given that an understanding of the mode of action of secreted proteins depends on a detailed knowledge of their subcellular localization, the present study investigates the distribution of BDNF in the adult hippocampus at both light microscopic and ultrastructural levels using three lines of transgenic animals: (1) a BDNF knockin line allowing the use of antibodies directed against a tagged version of the BDNF gene (Matsumoto et al., 2008), (2) a line conditionally lacking BDNF in adult neurons allowing for the specificity control of BDNF antibodies (Rauskolb et al., 2010), and (3) a mutant line displaying seizure episodes that are accompanied by markedly elevated levels of BDNF (Heyden et al., 2011). Antibodies against the BDNF prodomain were also used as an independent means of localizing BDNF as well as to generate information about the status of its cleaved prodomain (described hereon as the pro-peptide).

**Results**

**Antibodies to BDNF and pro-BDNF reveal similar staining patterns**

Hippocampal sections prepared from 8-wk-old animals were incubated either with the monoclonal BDNF antibody Mab#9 (anti-BDNF; Fig. 1 B) or with polyclonal antibodies recognizing the BDNF prodomain (anti–pro-BDNF; Fig. 1 H). Tissues from age-matched mice engineered to delete BDNF from neurons (cbdnf ko; Rauskolb et al., 2010) were used as a negative CON (Fig. 1, C, D, I, and J). In addition, sections from a knockin mouse line expressing Bdnf-Myc (Fig. 1 E; Matsumoto et al., 2008) were incubated with Myc antibodies (anti-Myc), with wild-type (WT) tissue used as a CON (Fig. 1, F and G). These three unrelated antibodies yielded strikingly similar staining patterns (Fig. 1 B, E, and H). In particular, BDNF-, pro-BDNF-, and Myc-immunoreactivity (IR) were each most prominently distributed in the cell bodies and axon terminals of the mossy fiber projection pathway, whereas the layers comprising the CA1 area were only weakly stained, especially in the septal hippocampus.
BDNF-IR is detected in subsets of neurons

Low-power examination of BDNF-IR in the dentate gyrus (DG) revealed a subset of immunopositive granule cells in the supragenular and infrapyramidal blades (Fig. 2 A). BDNF-IR varied in intensity among the labeled cells, with staining concentrated in the somal apex (Fig. 2 A, inset). Anti–pro-BDNF staining was confined to exactly the same subset of granule cells containing BDNF-IR (Fig. 2, B and C), with somal pro-BDNF–IR also concentrated at the apex (Fig. 2 B, inset). In addition, the hilar region, which contains mossy fiber collateral axons of the granule cells, was intensely stained (Fig. 2 A–C). In the CA3 region, a subset of pyramidal neurons also showed both BDNF-IR (Fig. 2, D and F) and pro-BDNF–IR (Fig. 2, E and F). High-resolution examination of sections labeled with anti-Myc and anti–pro-BDNF, along with antibodies against the Golgi matrix protein GM130, revealed Myc-IR throughout the soma and the initial dendritic segments (Fig. 2 G), whereas pro-BDNF–IR showed a similar, albeit more punctate, distribution (Fig. 2 H). Comparison with GM130-IR showed pro-BDNF–positive puncta closely associated with the Golgi apparatus (Fig. 2, I and J). In line with this, anti–pro-BDNF immunogold labeling localized the protein to the Golgi complex of CA3 somata (Fig. 2 K). In CA1, BDNF and pro-BDNF containing was also detected in a small number of pyramidal neurons in temporal hippocampus sections (unpublished data).

BDNF-IR and pro-BDNF–IR are both detected in presynaptic terminals

The granule cells give rise to mossy fiber axons, whose targets include the complex spines on proximal dendrites of CA3 neurons. Mossy fibers project through and terminate in stratum lucidum (SL) and are characterized by prominent specialized endings known as mossy fiber boutons (MFBs). Accordingly, strong BDNF-IR and pro-BDNF–IR were observed within SL (Figs. 1 B, E, and H and 2 D–F). Using high-resolution confocal microscopy, both BDNF-IR and pro-BDNF–IR were found to be colocalized to the same subset of MFBs (Fig. 3, A–C).

Additional markers were then applied to identify the type of vesicles containing BDNF-IR and to compare the distribution of BDNF with other peptides known to be anterogradely transported by granule cells. As expected, BDNF-IR did not colocalize with the synaptic vesicle markers synaptophysin (SYP; Fig. 3, D and E) or VGLUT-1 (Fig. 3, F and G). We then tested possible colocalization with Met-enkephalin (Met-enk), an opioid peptide also derived from a larger precursor protein (DCVs; Cheng et al., 1995). In a small proportion of granule cells and their axons, Met-enk–IR was detected throughout the soma and initial dendritic segment (Fig. 3 I). Although Met-enk–positive granule cells invariably coexpressed BDNF-IR (Fig. 3 H), the immunoreactive signals of these two precursor-derived molecules remained separate, suggesting that they do not reside together in the same secretory vesicles (Fig. 3, J and K–M). Similar conclusions were reached with cholecystokinin (CCK), a neuropeptide transported along the mossy fiber projection pathway of the ventral mouse hippocampus (Gall et al., 1986). Double labeling with anti-CCK and -BDNF revealed complete segregation of the two peptides within MFBs (Fig. 3, N–P).
Presynaptic BDNF labeling was also observed in the CA1 region, whereby in stratum radiatum (SR), thin varicose processes labeled with anti-BDNF (Fig. 3, Q–S) and anti–pro-BDNF (not depicted) were sparsely distributed throughout the neuropil, likely corresponding to presynaptic Schaffer collateral axons originating from BDNF-positive CA3 neurons. Importantly, BDNF-IR did not colocalize with the postsynaptic marker synaptopodin (synpo; Fig. 3, R and S).

**BDNF and pro-BDNF antibodies label secretory vesicles in presynaptic terminals**

Ultrathin sections of SL were then examined at 2,000-fold magnification after immunogold labeling with BDNF and pro–BDNF antibodies. MFBs were identified based on their typical morphological characteristics, namely a high density of synaptic vesicles, numerous synaptic contacts with CA3 complex spines, nonsynaptic puncta adherentia at dendritic shafts, and a relatively large surface area. A subset of MFB profiles labeled with anti-BDNF (Fig. 4 A), anti-Myc (not depicted), or anti–pro-BDNF (Fig. 4 B) contained distinct aggregates of gold grains; at 10,000-fold magnification, these gold clusters were found to be associated with large vesicles encompassed by an electron-dense membrane. Although the vesicles were sometimes masked by gold grains, fortuitous grain distribution occasionally revealed an electron-dense core (Fig. 4, A and B, insets). Within MFB profiles of cbdnf ko sections, gold grains were never specifically associated with any type of organelle (Fig. 4, C and D). To assess the density of immunogold particles in MFB profiles, gold clusters and single gold grains were quantified in anti-BDNF and anti–pro-BDNF–labeled tissues.
and compared between sections from pooled WT/Bdnf-Myc mice and cbdnf ko mice. Anti-BDNF–labeled MFB profiles showed a mean density of 2.67 ± 0.35 clusters/µm² in WT/Bdnf-Myc animals compared with 0.38 ± 0.12 clusters/µm² (P < 0.005) in cbdnf ko profiles (Fig. 4 E). Moreover, single gold grain densities were significantly reduced in cbdnf ko MFB profiles, with 5.63 ± 0.94 grains/µm² compared with 21.99 ± 2.57 grains/µm² in WT/Bdnf-Myc (P < 0.005; Fig. 4 F).

Similarly, pro-BDNF immunogold labeling showed a mean density of 2.01 ± 0.43 clusters/µm² and 27.44 ± 8.21 single grains/µm² in MFB profiles from WT/Bdnf-Myc animals, whereas in comparison, MFB profiles from cbdnf ko animals showed significant reductions in cluster (0.14 ± 0.10/µm²; P < 0.05; Fig. 4 E) and single grain (5.82 ± 2.06/µm²; P < 0.05; Fig. 4 F) densities, respectively.

Although BDNF immunogold labeling was mostly concentrated in presynaptic terminals, labeled vesicles were also occasionally observed within unmyelinated axon segments in SL (Fig. 5 A), which in fortuitous sections could be seen to give rise to giant MFBs (Fig. 5 B).

Ultrathin sections of SR (CA1) labeled with anti-BDNF and anti–pro-BDNF immunogold were also examined, and, as expected, large cluster-labeled secretory vesicles were observed within small axon terminals (Fig. 5 C). Depending on the proximal distal level of SR, these infrequent labeled boutons likely correspond to Schaffer collateral terminals or entorhinal terminals. No such labeled terminals were observed in cbdnf ko sections (Fig. 5 D).

**BDNF-IR and pro-BDNF-IR are not detected in dendrites**

Next, we examined the possible localization of BDNF in dendrites by analyzing sections double labeled with anti-Myc and anti–microtubule-associated protein–2 (MAP-2) (MAP-2). Both in BDNF-positive granule cells and CA3 neurons, Myc-IR only extended as far as the initial dendritic segments, and there was no evidence of colocalization in SL (Fig. 6, A–C). Sections were alternatively labeled with antibodies to Arc/Arg3.1, an immediate early gene product up-regulated in somata and dendrites during elevated synaptic activity (Lyford et al., 1995). Confocal scanning revealed that the majority of granule cells labeled with BDNF-IR and pro-BDNF-IR also expressed Arc/Arg3.1-IR (Fig. 6, D–G). Although Arc/Arg3.1-IR was seen throughout the soma and dendritic arbor, strong coexpression of BDNF-IR and pro–BDNF-IR was confined to the cell soma (Fig. 6, D–G).

In ultrathin sections prelabeled either with anti-BDNF or anti–pro-BDNF immunogold, gold grains were sparsely distributed within dendritic profiles (Fig. 6, H and K). When profiles were thoroughly scrutinized for specifically labeled vesicles or endosomes, none was found to be stained above the background levels observed in dendrites from cbdnf ko sections (Fig. 6, I and L). Gold grain quantification of anti-BDNF–labeled sections (Fig. 6 J) revealed a mean density of 3.56 ± 0.45 grains/µm² in WT/Bdnf-Myc mice versus 4.72 ± 0.91 grains/µm² in cbdnf ko mice (P = 0.81). Similarly, in anti–pro-BDNF–stained sections, mean densities were comparable between WT/Bdnf-Myc mice (4.69 ± 1.09 grains/µm²) and cbdnf ko mice (4.08 ± 2.02 grains/µm²; P = 0.31; Fig. 6 M).

**Localization of BDNF-IR and pro-BDNF-IR in Bsn mutants**

Next, we examined the hippocampus of mice lacking the presynaptic protein Bassoon, as these mutants develop episodic generalized seizures (Altrock et al., 2003) and have enlarged cortices and hippocampi (Agnen et al., 2007). Concurrent with the development of seizures, BDNF protein levels become significantly higher than those measured in adult CON littermates (see Fig. 10 C; Heyden et al., 2011). Whereas Bassoon (Bsn) mutants showed the typical distribution pattern of BDNF-IR and pro–BDNF–IR, a dramatic increase in staining intensity largely confined to the neuropil was observed (compare CON in Fig. 7 [A–D] with Bsn in Fig. 7 [E–H]). In contrast, granule cell
bodies from Bsn mutants did not show increases in anti-BDNF (Fig. 7 I) or anti–pro-BDNF (Fig. 7 J) staining intensities, although a much higher proportion of cells was labeled in comparison with CON tissues. Granule cell dendrites in the molecular layer remained unlabeled in Bsn mutants, whereas in the CA3 region, the stark increase in BDNF-IR (Fig. 7 G) and pro-BDNF–IR (Fig. 7 H) was confined to SL. Closer examination revealed intense presynaptic labeling in MFB profiles (Fig. 7, K and L), which was confirmed by a lack of colocalization with the postsynaptic markers synpo (Fig. 7 K) and MAP-2 (Fig. 7 L), respectively.

Enhanced BDNF staining in Bsn mutants was not only confined to the granule cell–CA3 projection pathway. In the CA1 region, where BDNF-IR can usually only be detected at high magnification (compare Fig. 1 with Figs. 3 Q and 5 D), a conspicuous band of punctate BDNF-IR and pro-BDNF–IR was observed at the border of SL and stratum lacunosum–moleculare (SL-M; Fig. S1, A–C), corresponding to a region known to harbor fibers from the entorhinal cortex (Amaral and Lavanex, 2006). Importantly, these increased signals colocalized (Fig. S1 D), showing no overlap with either synpo-IR (Fig. S1 E) or glial fibrillary acidic protein (GFAP)–IR (Fig. S1 F), which label dendritic spines and reactive astrocytes, respectively. This suggests that entorhinal neurons also represent a possible presynaptic source of BDNF for CA1 neurons.

Immunogold-labeled sections from both WT and Bsn mutants were then examined at 2,000-fold magnification. In comparison with CON animals (Fig. 8, A and B), a higher number of labeled MFB profiles containing more BDNF-positive DCVs was observed in sections from Bsn mutants (Fig. 8, C and D), with clusters accumulated at the synaptic membrane. This was confirmed by quantification, with anti-BDNF–labeled MFBs containing a significantly higher density of gold clusters (1.7 ± 0.3 clusters/µm²; Fig. 8 E) and grains (14.4 ± 2.5 grains/µm²; Fig. 8 F) compared with CONs (0.8 ± 0.01 clusters/µm² with P < 0.05 and 6.9 ± 0.5 grains/µm² with P < 0.05, respectively). Similarly, anti–pro-BDNF–labeled profiles from Bsn mutant brains also displayed a higher density of gold clusters (2.02 ± 0.27 clusters/µm²; Fig. 8 E) and grains (17.2 ± 2.8 grains/µm²; Fig. 8 F) compared with CON profiles (0.96 ± 0.04 clusters/µm² with P < 0.005 and 10.1 ± 0.9 grains/µm² with P < 0.05, respectively); these relatively low increases in mean cluster densities are a result of the larger areas of MFB profiles in Bsn mutants.

Gold grain distribution and density in dendrites were also compared between Bsn mutant (Fig. 9, C and D) and CON tissues (Fig. 9, A and B). Dendritic profiles were again scrutinized for evidence of labeled vesicles, but none was found in tissues from either group. Density measurements revealed background values in Bsn mutant mice similar to those observed in CON mice, both in tissues labeled with anti-BDNF (5.4 ± 0.4 grains/µm² for Bsn mutant vs. 5.7 ± 0.5 grains/µm² for CON; P = 0.66; Fig. 9 E, left) and anti–pro-BDNF (5.1 ± 0.1 grains/µm² for Bsn mutant vs. 6.3 ± 0.3 grains/µm² for CON; P = 0.12; Fig. 9 E, right). Increased labeling density was not detected in the extracellular space or in nonneuronal cell types such as astrocytes.

Verification of background immunogold labeling
To determine whether background gold labeling is evenly distributed over different subcellular compartments, we extended the quantitative gold grain analysis to dendritic spine profiles and myelinated axon profiles in SL, as they are devoid of clustered-labeled organelles. Quantification of single gold grains overlying these profiles revealed that the density of background gold labeling depends on the type of subcellular compartment (Table 1). The mean values for gold grain densities in spine and myelinated axon profiles did not differ between WT/Myc versus cbdnf ko or WT versus Bsn mutant tissues. Therefore, spines and myelinated axons from WT mice do not exhibit anti-BDNF and anti–pro-BDNF immunogold labeling above the background levels observed in cbdnf ko tissues, nor do they contain specifically labeled organelles.

Biochemical detection and quantification of the BDNF pro-peptide
The identity of the molecules recognized by the BDNF antibodies in the immunochemistry experiments was then determined...
using hippocampal lysates from WT and mutant mice (cbdnf ko and Bsn). Immunoprecipitates were analyzed by Western blotting (WB) and probed with either the BDNF polyclonal antibody N-20 or the monoclonal antibody 5H8, which recognizes an epitope in the prodomain of BDNF (Fig. 1 A). These experiments revealed the presence not only of proBDNF (<32 kD) but also of the much more abundant pro-peptide (<17 kD; Fig. 10 A; also see Fig. 1 A). Both signals were absent in lysates from cbdnf ko animals (Fig. 10 A). Quantification of the corresponding signal intensities revealed that the ratio of pro-peptide versus pro-BDNF is ~10.3 ± 2.0 (n = 3; Fig. 10 B), similar to that of BDNF versus pro-BDNF (11 ± 2.0; n = 3; Fig. 10, B and C). A similar analysis of hippocampal lysates from Bsn mutants confirmed an approximate threefold increase each in BDNF, its pro-peptide, and pro-BDNF in mutant tissues (Fig. 10 C). These results also indicate that the respective ratios of BDNF and the pro-peptide versus pro-BDNF are similar to what is observed in WT animals. Of note is that the consistent detection of BDNF pro-peptide was only possible after glutaraldehyde fixation of the transfer membrane (see Materials and methods). Furthermore, under the lysate and incubation conditions used, no measurable proteolysis of recombinant pro-BDNF (500 pg) added to the lysates at the beginning of the extraction procedure could be detected (Fig. S2). The recovery of added recombinant pro-BDNF was 102.2 ± 5.8% (n = 4).

Discussion

Our study reveals that in the adult hippocampus, BDNF and its cleaved pro-peptide are stored in large DCVs located in the presynaptic terminals of excitatory neurons. Both in WT and Bsn mutant mice, BDNF and its pro-peptide are stored at roughly equimolar ratios and are ~10-fold more abundant than pro-BDNF in hippocampal lysates. Together with the lack of any detectable BDNF staining in dendrites or spines, our results provide direct support for an anterograde mode of action for BDNF in the intact CNS. They also offer a morphological substrate for recent findings indicating that the release of endogenous BDNF accounts for some of the rapid calcium transients observed at synaptic sites on dendrites of CA3 neurons (Lang et al., 2007).
Immunohistochemical localization of BDNF in the CNS

The specificity of BDNF immunostaining in the adult brain is very difficult to ascertain, as Bdnf knockout animals die before reaching maturity, a fact that complicates the interpretation of previous immunohistochemical studies (Wetmore et al., 1991; Dugich-Djordjevic et al., 1995; Schmidt-Kastner et al., 1996; Altar et al., 1997; Conner et al., 1997; Yan et al., 1997; Luo et al., 2001; Danzer and McNamara, 2004; Tongiorgi et al., 2004; Zhou et al., 2004; Agassandian et al., 2006; Avwenagha et al., 2006, Salio et al., 2007). Our study deals with the mature hippocampus, as much of the current interest in BDNF relates to its role in the adult brain, and the staining specificity of our BDNF monoclonal antibody was verified with tissues from age-matched mice lacking BDNF in the CNS (Kolbeck et al., 1999; Rauskolb et al., 2010). The choice of BDNF antibody was also important, as none of the commercially available BDNF antibodies we tested yielded specific staining (unpublished data). In addition, a mouse line was used in which Bdnf was replaced with a Myc-tagged version of the gene (Matsumoto et al., 2008), allowing additional control experiments to be performed with anti–human Myc antibodies on WT tissue sections. We note that a recent light microscopy study that also used a knockin strategy to tag endogenous BDNF came to a conclusion very similar to our own, revealing identical labeling of the mossy fiber pathway with antibodies directed against either the HA epitope used to tag BDNF or the BDNF prodomain (Yang et al., 2009). However, our biochemical results (see later part of Discussion) now indicate that pro-BDNF antibodies primarily detect the BDNF pro-peptide, which is present at much higher levels than uncleaved pro-BDNF. The pro-BDNF antibodies further validate our localization data, as they recognize an epitope unrelated to those detected by the antibodies to BDNF or Myc but similarly label presynaptic large DCVs. These organelles are well known to be involved in the regulated secretion of many other neuronal signaling peptides including Met-enk (Cheng et al., 1995), which was also used here as a presynaptic marker. DCVs are typically found in presynaptic terminals but rarely in postsynaptic structures such as dendrites or spines, with the notable exception of the DCVs containing the neuropeptides vasopressin and oxytocin, found in the dendrites of specialized neurosecretory cells (Kennedy and Ehlers, 2011).

Using cultured cells to study the cell biology of BDNF

Numerous previous studies have used cultured hippocampal neurons to study the storage and release of BDNF (Haubensak et al., 1998; Hartmann et al., 2001; Wu et al., 2004; Adachi et al., 2005; Brigadski et al., 2005; An et al., 2008; Dean et al., 2009; Matsuda et al., 2009). They all come to the conclusion that BDNF is stored in and released from both axonal and dendritic compartments, raising the question of what could underlie the apparent discrepancy with our findings. Although the majority of these studies utilize neurons transfected with a cDNA encoding a fluorescently tagged form of BDNF, some of the conclusions are also based on the detection of endogenous BDNF in vitro. However, cultured embryonic neurons grow in a very different environment compared with neurons in vivo, with fewer and less intimate cell–cell interactions between neighboring neurons and astrocytes, an incomplete ECM, and a lack of laminar input. In vivo, the development of neuronal circuits is spatially tightly regulated (Frotscher et al., 2000), whereas in vitro, synapses seem to form randomly. As a result of these cell-extrinsic differences, it is conceivable that cultured neurons fail to sort cargo-loaded vesicles as strictly as they do in vivo (Mellman and Nelson, 2008). In addition, we note that cultured neurons express significantly higher levels of BDNF than are detectable even in adult hippocampal extracts (unpublished data).
BDNF signaling have inferred the translation of BDNF mRNA in dendrites (Tanaka et al., 2008), it has not been possible thus far to assess directly whether dendritic BDNF mRNAs are translated locally. Thus, it is of particular significance that our experiments revealed a complete lack of specific BDNF protein signals in dendrites not only in WT but also in Bsn mutant animals. Although our experiments cannot rule out that functionally relevant quantities of BDNF may be released from dendrites, they indicate that, at present, there is no structural basis for this speculation.

With regard to the biochemistry of BDNF, initial studies suggested that the most abundant, or indeed the only detectable, translation product in adult brain extracts was uncleaved pro-BDNF (Chen et al., 2005; An et al., 2008). However, a signal of similar size and intensity to pro-BDNF can readily be detected in mice lacking BDNF in the CNS (Matsumoto et al., 2008). Furthermore, pulse-chase experiments showed that pro-BDNF is a short-lived intermediate that is rapidly processed intracellularly (Matsumoto et al., 2008). BDNF, like all other neurotrophins, is initially translated as a glycosylated precursor protein, presumably allowing the proper folding and disulfide bridging of the mature protein (Leibrock et al., 1989; Rattenholl et al., 2001). Accordingly, we detected specific pro-BDNF–IR in the

**BDNF biosynthesis and processing**

Although in situ hybridization experiments have long established that BDNF mRNA can be unambiguously detected in the cell bodies of excitatory neurons (Hofer et al., 1990; Isackson et al., 1991), some studies have also suggested its presence in dendrites (Tongiorgi et al., 1997, 2004; An et al., 2008) and its active transport upon stimulation (Tongiorgi et al., 1997, 2004; Chiaruttini et al., 2009; Baj et al., 2011; Louhivuori et al., 2011; Wu et al., 2011). However, these experiments revealed that BDNF mRNA is predominantly detected in the most proximal part of the dendrite, whereas other actively transported mRNAs can be detected in dendrites hundreds of micrometers away from the soma, as is the case for the Arc/Arg3.1 (Lyford et al., 1995), CaMKIIα (Burgin et al., 1990; Miller et al., 2002), or dendrin (Link et al., 1995) mRNAs. This leaves the possibility that BDNF mRNA localizes to proximal dendrites by diffusion rather than active transport. Selective trafficking has also been difficult to assess, as activity-dependent transcription augments the intensity of the proximal mRNA signal, allowing more distal diffusion of the in situ hybridization enzymatic reaction product (Tongiorgi et al., 1997, 2004). Although elegant experiments aimed at blocking protein synthesis and interfering with

**Figure 8.** Higher density of BDNF-positive DCVs in MFBs of Bsn mutant animals. (A and B) Electron micrographs of MFBs from CON sections prelabeled with BDNF (A) or pro-BDNF (B) immunogold. Gold cluster-labeled secretory vesicles were occasionally observed in a subset of terminals (arrows), sp, dendritic spine. (C and D) In MFBs from Bsn mutants, there was an increase in the number of cluster-labeled vesicles (arrows) as well as single gold grains, both in the case of BDNF (C) and pro-BDNF (D) immunogold. den, dendrite. (E and F) This was confirmed by quantification. Error bars represent SEM. Bars, 500 nm.

**Figure 9.** BDNF is not targeted to dendrites in Bsn mutants. (A–E) Comparison of dendrites (den) in SL in CON (A and C) versus Bsn mutant tissues (B and D) revealed a lack of difference in the density of gold grains, both in the case of BDNF immunogold (E, left) and pro-BDNF immunogold (E, right). Also note the general absence of DCVs within dendritic profiles of both CON and Bsn mutant animals. Error bars represent SEM. Bars, 500 nm.
Table 1. Comparative gold grain densities in subcellular compartments devoid of cluster-labeled organelles

| Subcellular profile | Anti-BDNF immunogold | Anti-pro-BDNF immunogold |
|---------------------|-----------------------|--------------------------|
|                     | WT/Myc | cbdnf ko | CON | Bsn | WT/Myc | cbdnf ko | CON | Bsn |
| Dendrite\(^a\)      | n = 3  | n = 3    | n = 4 | n = 4 | n = 3  | n = 3    | n = 4 | n = 4 |
| 3.6 ± 0.5           | 4.7 ± 0.9 | 6.9 ± 0.9 | 6.6 ± 0.7 | 4.7 ± 1.6 | 4.1 ± 1.5 | 6.3 ± 0.3 | 5.1 ± 0.1 |
| Spine               | 19.3 ± 2.1 | 18.4 ± 3.8 | 15.0 ± 1.4 | 13.1 ± 0.9 | 18.2 ± 1.2 | 20.3 ± 4.5 | 13.8 ± 0.6 | 14.3 ± 1.6 |
| Myelinated axon     | 9.6 ± 1.8 | 11.6 ± 0.7 | 8.3 ± 0.5 | 8.1 ± 1.2 | 12.2 ± 0.8 | 10.7 ± 0.7 | 8.5 ± 1.1 | 10.1 ± 1.5 |

Two-sided \(t\) tests for all profiles in both anti-BDNF immunogold and anti-pro-BDNF immunogold were \(P > 0.05\). All values expressed as the group mean value ± SEM \(n\) value correspond to the number of animals examined per experiment. Subacellular profiles are represented as no. of grains/\(\mu m\(^2\).

\(^a\)Gold grain densities in dendrites are also presented in graph form in Fig. 6 (WT/Myc vs. cbdnf ko) and Fig. 9 (CON vs. Bsn mutant).

Golgi apparatus of dentate granule cells and CA3 neurons but not in other organelles such as lysosomes or endosomes. Although the intracellular site of pro-BDNF cleavage and the participating proteases are yet to be identified, the existence of the BDNF pro-peptide in brain lysates has not been previously reported (see Fig. 10). This pro-peptide has hitherto escaped detection presumably because it is readily washed away from blotting membranes. This loss could be prevented by fixing the membranes with glutaraldehyde after electrophoretic transfer (Karey and Sirbasku, 1989).

Although we observed that prolonged stimulation of cultured neurons results in the cosecretion of BDNF and its pro-peptide (unpublished data), the biological significance of this corelease is unclear.

**BDNF detection in the hippocampus of Bsn mutants**

Mutant mice expressing a truncated version of the large presynaptic protein Bassoon develop spontaneous epileptic seizures for reasons that are only partially understood (Altrock et al., 2003). In line with the well-established link between excitatory neuronal activity and Bdnf transcription, BDNF protein levels were found to increase in parallel with, but not precede, the development of seizure episodes (Heyden et al., 2011). It seems unlikely that Bassoon is directly involved in the increase in BDNF levels, as a mutation in the potassium channel Kv1.1 that also leads to seizure activity is accompanied by a marked increase in BDNF mRNA and megencephaly (Diez et al., 2003; Lavebratt et al., 2006), as is the case for the Bsn mutation (Angenstein et al., 2007). Blocking activity with carbamazepine in the Kv1.1 mutant prevents the development of megencephaly and restores BDNF mRNA to normal levels (Lavebratt et al., 2006). Despite the marked increase in BDNF and its pro-peptide in the Bsn mutant, both immunoreactive signals remained confined to presynaptic terminals, with no detectable staining in either granule or pyramidal cell dendrites or glial cells (Bergami et al., 2008). In comparison with WT animals, the only remarkable change observed in the Bsn mutant was an increase in the density of BDNF-positive DCVs in MFBs. These observations are in line with earlier studies in the rat, in which chemically induced seizure activity led to an increase in presynaptic BDNF staining (Vezzani et al., 1999; Scharfman et al., 2002; Danzer and McNamara, 2004). Despite the substantially higher BDNF levels, we did not detect any BDNF staining in the extracellular space, suggesting that after its release, BDNF becomes rapidly diluted by diffusion as well as by cellular reuptake, thus preventing accumulation to a degree that would allow immunohistochemical detection.

**BDNF and hippocampal mossy fibers**

The hippocampal mossy fiber projection stands out as the most heavily stained structure in the adult CNS using either BDNF or pro-BDNF antibodies (Fig. 1). This pathway is thought to exhibit presynaptic long-term potentiation (LTP; Nicoll and Schmitz, 2005) and to be important for pattern completion as well as for the storage and recall of information.

---

**Figure 10. Biochemical detection of BDNF, its pro-peptide, and pro-BDNF.** IP of hippocampal lysates (500 µg) was performed either with an anti–pro-BDNF antiserum or with the BDNF antibody followed by WB with the antibodies indicated. (A) Both pro-BDNF and its pro-peptide were detected in WT (first lane) but not in cbdnf ko samples (second lane). Similar results were obtained using the pro-BDNF antibody AN-03 in IP and/or WB (not depicted). HIP, hippocampus; IB, immunoblot; nonglyc., nonglycosylated; rec., recombinant. (B) Note that the ratio of pro-peptide to pro-BDNF is similar to that of BDNF and pro-BDNF (n = 3). Error bars represent the SEM of the three samples measured. (C) IP/WB analysis of hippocampal lysates from Bsn mutants reveals a relatively similar increase (approximately threefold) in the levels of BDNF and pro-BDNF (left, second lane) as well as pro-peptide and pro-BDNF (right, second lane) compared with CON tissues (left and right, first lanes). Recombinant BDNF pro-peptide and cleavage-resistant pro-BDNF were used as molecular mass markers.
polyclonal antibody (anti–pro-BDNF; #ANT-006, batch AN03; Alomone Labs) raised against the prodomain of BDNF protein (see Fig. 1 A). To determine whether the signals obtained with anti–pro-BDNF could also be observed with other antibodies against the prodomain of BDNF, we tested a commercially available mouse monoclonal pro-BDNF antibody (anti–pro-BDNF–Mab5SH; SC-65514; Santa Cruz Biotechnology, Inc.). The staining pattern produced by this antibody (as specified in the figure legends) was comparable with the results obtained with the polyclonal anti–pro-BDNF. Presynaptic components were detected with mouse monoclonal antibodies against the vesicular glutamate transporter 1 (anti–VGLUT-1; #135511; Synaptic Systems) and rabbit polyclonal antibodies against SYP (anti–SYP; Santa Cruz Biotechnology, Inc.); for postynaptic labeling, mouse monoclonal antibodies against MAP-2 (anti-MAP-2; #M2320; Sigma-Aldrich), rabbit polyclonal antibodies against the spine apparatus protein synaptojanin (anti-syn, #59567; Sigma-Aldrich), and guinea pig polyclonal antibodies against activity-related cytoskeletal protein (anti–Arc/Arg3.1; #156005; Synaptic Systems) were used. A mouse monoclonal antibody against the 130-kD Golgi matrix protein (anti–GM130) was used for the detection of the Golgi complex (#610822; BD). For labeling of neuropeptides within the mossy fiber projection, rabbit polyclonal antibodies against Met-enkephalin (anti–Met-enk; Millipore) and CCK (anti–CCK; #P06307; Millipore) were used. Astrocytes were detected with a guinea pig polyclonal antibody serum against GFAP (anti–GFAP; #031223; Advanced ImmunoChemical Inc.).

Immunofluorescence

As preliminary staining experiments with anti–Myc antibodies yielded high levels of nonspecific staining in sections from WT mice, the Myc antibody solution was substituted with WT sections and the antibody subsequently used to stain sections from Bdnf-Myc and WT mice. Except for the first blocking step, the staining protocol for all antibodies was identical, and TBS, pH 7.4, was used throughout to wash the sections. For staining with polyclonal antibodies, sections were blocked with 20% normal donkey serum in TBS for 1 h; for staining with monoclonal antibodies, sections were blocked with 3% M.O.M (Mouse-on-Mouse blocking serum; Vector Laboratories) in TBS for 1 h. Primary antibodies were diluted in a solution of 3% BSA, 2% normal donkey serum, and 0.02% Triton X-100 in TBS to yield the following final concentrations/dilutions: 10 µg/ml anti-BDNF, 0.4 µg/ml anti-NR2B, 1.33 µg/ml anti–pro-BDNF, 3.3 µg/ml anti–VGLUT-1, 4 µg/ml anti–SYT, 0.9 µg/ml anti–GM130, 2 µg/ml anti–Arc/Arg3.1, 4 µg/ml anti–MAP-2, 1.2,000 anti-synaptojanin, 3 µg/ml anti–Arc/Arg3.1, 1:2,000 anti–CCK, and 1:1,000 anti–GFAP. Sections were incubated for at least one night at 4°C in either one or more of these primary antibodies. For fluorescent signal detection, the following Alexa Fluor–conjugated secondary antibodies were used: donkey anti–rabbit-488 or -555, donkey anti–goat-555 or -488, donkey anti–mouse-555 or -647, and donkey anti–guinea pig–Cy3 (all purchased from Millipore). Labeled sections were mounted onto glass slides and coverslipped with fluorescent mounting medium (Dako).

Confocal microscopy

Slide-mounted sections of immunolabeled hippocampi were viewed on an inverted microscope (Axiovert 200; Carl Zeiss) equipped with Plan Apochromat 20× (0.75 NA) or 63× (all differential interference contrast; 1.4 NA) objectives and attached to a spectral confocal laser system (LSM510; Carl Zeiss) powered by LSM 4.2 Meta software (Carl Zeiss). The tissue was scanned at room temperature with 488-, 543-, and 633-nm laser lines to detect the Alexa fluorophores 488, 555, and 647, respectively, and high-resolution images of optical sections (z slices) were captured using sequential line (mean of four) scanning. Colocalization of two or three fluorophores was quantitatively assessed in the x, y, and z planes of each optical section. Maximal projection images of confocal z series (stacks) were generated where indicated in the figure legends. Minimal adjustments to image contrast and intensity were made in Photoshop CS (version 8.0.1; Adobe) using the levels or contrast/brightness functions. Images were arranged and annotated using CorelDRAW 12 software (Corel Corporation).

Immunogold EM

Immunogold prelabeling. 50-µm vibratome sections of perfusion-fixed hippocampi from WT, Bdnf-Myc, cbrdfl ko, and Bsn mutant mice were thoroughly washed in 50 mM TBS, pH 7.4, and immersed for 2 h in a cryoprotecting solution (25% sucrose and 10% glycerol in 0.05 M PB) before being snap frozen in liquid nitrogen–cooled isopentane. After immediate thawing and washing in 0.1 M PB, sections were incubated for 1 h in blocking solution (5% M.O.M. in TBS for anti–pro-BDNF staining and 20% normal goat serum in TBS for anti–pro-BDNF and anti–Myc staining)
followed by one to three nights at 4°C in a 3% BSA/TBS solution containing either 20 µg/ml anti-BDNF, 1.33 µg/ml anti-pro-BDNF (AN-03), or 0.4 µg/ml anti-Myc antibody. After thorough washing in TBS, the sections were incubated overnight at 4°C in a 3% BSA/TBS solution containing either 1.4 nm of gold-conjugated goat anti-mouse, goat anti-rabbit, or rabbit anti-goat IgG. Sections were rinsed in TBS and then fixed for 10 min on a 1% glutaraldehyde solution. Tissue-bound gold particles were enlarged using an Ultramat silver catalyzer kit (HQ Silver, Nanoprobes). The sections then underwent osmification for 40 min in a solution of 0.5% OsO4 and 6.86% sucrose in 0.1 M PB.

Embedding. After osmification, sections were washed in 0.1 M PB followed by 50% ethanol (EtOH). The tissue was then incubated in 1% uranyl acetate in 70% EtOH for 35 min followed by 10-min dehydration steps in increasing grades of EtOH. After washing in propylene oxide, the tissue was embedded in Durcupan (Fluka). Ultrathin sections (60 nm) of selected hippocampal areas (DG, CA3, and CA1) were cut and mounted on formvar-coated nickel grids. Sections were viewed and examined in an electron microscope (LEO 906E; Carl Zeiss).

Quantification of immunogold staining
Gold-labeled sections from WT, Bdnf-Myc, cbdnf ko, and Bsn mutant animals were first examined in the electron microscope at magnifications ranging from 6,000 to 12,000×, and areas containing labeled structures in SL of CA3 were then photographed using a dual-speed charge-coupled device camera (TR5 SharpEye; Troendle) and analySIS acquisition software (Soft Imaging System; Olympus). MFBs were unequivocally identified based on their well-defined morphology (see criteria in Results). An organelle in an MFB was deemed labeled if associated with at least four gold grains (described in this study as a cluster). MFBs containing labeled organelles were then selected for quantitative analysis, in which the number of clusters per bouton profile was manually counted, and the area and perimeter of each profile were simultaneously measured with cell®P software (Soft Imaging System). The number of clusters per bouton was then reexpressed as a density measurement (i.e., the number of clusters per µm2 of MFB). Although the rare gold clusters observed in cbdnf ko sections showed no association with any particular organelle, they were still included in the counts. In addition to cluster quantification, the respective number of single gold grains in MFBs from WT, Bdnf-Myc, cbdnf ko, and Bsn mutant tissues was also determined and ultimately expressed as a density measurement (i.e., the number of grains per µm2).

For quantification of gold labeling in dendritic profiles, an initial qualitative analysis of the tissue revealed a complete lack of cluster-labeled organelles, including large secretory vesicles and endosomes. Therefore, to compare gold grain density in dendrites from different groups of animals, the number of single gold grains per dendritic profile was counted and expressed in relation to the area of the corresponding profile (i.e., the number of grains per µm2).

Finally, to determine whether background gold grain labeling was equally distributed across different subcellular compartments, the quantitative gold grain analysis was extended to two additional compartments in SL, namely dendritic spine profiles and myelinated axon profiles. These two compartments were chosen on the basis of also being devoid of cluster-labeled organelles.

Immunoprecipitation (IP) and WB
Hippocampi were dissected from 8-wk-old WT, cbdnf ko, and Bsn mutant mice and then weighed and stored at −80°C. 10 vol/wt of radioligand-immunoprecipitation assay buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.2% Na deoxycholate) was added. To prevent proteolysis, freshly prepared protease inhibitors including a protease inhibitor cocktail (Roche), 10 µM 1,10-phenanthroline monohydrate, 10 mM 6-aminohexanoic acid, and 10 µg/ml aprotinin were added to the buffer. The tissues were sonicated, the homogenates centrifuged, and the supernatants collected. IP was performed with 500 µg of total protein using either the BDNF monoclonal antibody Mab#9 or a rabbit polyclonal antibody raised against the BDNF pro-peptide (Koshimizu et al., 2009) in the presence of protein G-agarose (Roche) according to the manufacturer’s instructions. After blotting, the proteins were fixed to the membrane with 0.5% glutaraldehyde for 30 min at room temperature and probed with either polyclonal rabbit anti-BDNF N20 (sc-546; Santa Cruz Biotechno-
logy, Inc.) or monoclonal mouse anti-pro-BDNF Mab5H8 (1:200) using an IP Western kit (GenScript) to eliminate nonspecific signals from protein G. Signal intensity measurements were performed using ImageJ software (National Institutes of Health). Recombinant WT and cleavage-resistant pro-BDNF (Fayard et al., 2005) as well as the mouse BDNF pro-peptide were produced in COS7 cells transfected with the corresponding cDNAs cloned in plasmid cytomegalovirus vectors. The concentrations of these proteins were then determined against known amounts of purified BDNF pro-peptide produced in Escherichia coli (Koshimizu et al., 2009). The COS7-derived cleavage-resistant pro-BDNF was used as a molecular mass marker, whereas WT pro-BDNF was used in recovery experiments with tissue lysates (Fig. S2).

Statistical analysis
All values are expressed as mean ± SEM. For BDNF and pro-BDNF immunogold quantification, 10–20 MFBs and 30–40 dendrites per WT, Bdnf-Myc, cbdnf ko, and Bsn mutant mice were analyzed, and mean values for each animal were calculated. Group means (poled WT/BDNF-Myc vs. cbdnf ko and WT vs. Bsn mutant) were compared using a Student’s t test. A probability of P < 0.05 was considered to be statistically significant.

Online supplemental material
Fig. S1 shows an increase in BDNF-IR and pro–BDNF-IR in area CA1 of the Bassoon mutant hippocampus. Fig. S2 shows the complete recovery of WT recombinant pro-BDNF from hippocampal lysates. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201201038/DC1.

We thank Drs. J. Kowalski and M. Sibbe for helpful statistical advice.

This work was supported by the Swiss National Foundation (31003A-124902/1), the German Research Foundation (SFB 780, project A4), and a Postdoctoral Research Fellowship [to S. Dieni] from the Alexander von Humboldt Foundation. M. Frotscher is a Senior Research Professor of the Hertie Foundation.

Submitted: 9 January 2012
Accepted: 15 February 2012

References
Adachi, N., K. Kohara, and T. Tsunomo. 2005. Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. BMC Neurosci. 6:42. http://dx.doi.org/10.1186/1471-2202-6-42
Agassandian, K., M. Gedney, and M.D. Cassell. 2006. Neurotrophic factors in the central nucleus of amygdala may be organized to provide substrates for associative learning. Brain Res. 1076:78–86. http://dx.doi.org/10.1016/j.brainsci.2006.01.009
Altar, C.A., N. Cai, T. Bliven, M. Juhaz, J.M. Conner, A.L. Acheson, R.M. Lindsay, and S.J. Wiegand. 1997. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature. 389:856–860. http://dx.doi.org/10.1038/39885
Altrock, W.D., S. tom Dieck, M. Sokolov, A.C. Meyer, A. Sigler, C. Bruckbusch, R. Fischo, K. tom Dieck, and H. Potschka et al. 2003. Functional inactivation of a fraction of excitatory synapses in mice deficient for the active zone protein bassoon. Neuron. 37:787–800. http://dx.doi.org/10.1016/S0896-6273(03)00088-5
Amaral, D., and P. Lavanex. 2006. Hippocampal Neuroanatomy. In The Hippocampus Book. P. Andersen, R. Morris, D. Amaral, T. Bliss, and J. O’Keefe, editors. Oxford University Press, Oxford. 37–114.
An, J.J., K. Bharani, G.Y. Liao, N.H. Woo, A.G. Lai, F. Vanesky, E.R. Torre, K.R. Jones, Y. Feng, B. Lu, and B. Xu. 2008. Distinct role of long 3’ UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell. 134:175–187. http://dx.doi.org/10.1016/j.cell.2008.05.045
Angenstein, F., H.G. Niessen, J. Goldschmidt, H. Lison, W.D. Altrock, E.D. Angenstein, F., H.G. Niessen, J. Goldschmidt, H. Lison, W.D. Altrock, E.D. Gundelangell, and H. Scheich. 2007. Manganese-enhanced MRI reveals structural and functional changes in the cortex of Bassoon mutant mice. Cereb. Cortex. 17:28–36. http://dx.doi.org/10.1093/cercor/bjh121
Awwenaga, O., M.M. Bird, A.R. Lieberman, Q. Yan, and G. Campbell. 2006. Patterns of expression of brain-derived neurotrophic factor and tyrosine kinase B mRNAs and distribution and ultrastructural localization of their proteins in the visual pathway of the adult rat. Neuroscience. 140:913–928. http://dx.doi.org/10.1016/j.neuroscience.2006.02.056
Baj, G., E. Leone, M.V. Chao, and E. Tongiorgi. 2011. Spatial segregation of BDNF transcripts enables BDNF to differentially shape distinct dendritic compartments. Proc. Natl. Acad. Sci. USA. 108:16813–16818. http://dx.doi.org/10.1073/pnas.1014618108
Baquet, Z.C., J.A. Gorski, and K.R. Jones. 2004. Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. J. Neurosci. 24:4250–4258. http://dx.doi.org/10.1523/JNEUROSCI.3920-04.2004
Fayard, B., S. Loeffler, J. Weiss, E. Vogelin, and A. Kriittgen. 2005. The secreted brain-derived neurotrophic factor precursor pro-BDNF binds to TrkB and p75NTR but not to TrkA or TrkC. J. Neurosci. Res. 80:18–28.  http://dx.doi.org/10.1002/jnr.20432

Frotscher, M., A. Drakew, and B. Heinrich. 2000. Role of afferent innervation and neuronal activity in dendritic development and spine maturation of fascia dentata granule cells. Cereb. Cortex. 10:946–951.  http://dx.doi.org/10.1093/cercor/10.9.946

Galimberti, I., N. Gogolla, S. Alberi, A.F. Santos, D. Muller, and P. Caroni. 2006. Long-term rearrangements of hippocampal mossy fiber terminal connectivity in the adult regulated by experience. Neuron. 50:749–763.  http://dx.doi.org/10.1016/j.neuron.2006.04.026

Galimberti, I., E. Bednarek, F. Donato, and P. Caroni. 2010. EphA4 signaling in juveniles establishes topographic specificity of structural plasticity in the hippocampus. Neuron. 65:627–642.  http://dx.doi.org/10.1016/j.neuron.2010.02.016

Gall, C., L.M. Berry, and L.A. Hodgson. 1986. Cholecystokinin in the mouse hippocampus: Localization in the mossy fiber and dentate commissural systems. Exp. Brain Res. 62:431–437.  http://dx.doi.org/10.1007/BF00238862

Goodman, L.J., J. Valverde, F. Lim, M.D. Geschwind, H.J. Fedoroff, A.L. Geller, and F. Hefti. 1996. Released regulation and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol. Cell. Neurosci. 7:222–238.  http://dx.doi.org/10.1006/mcne.1996.0017

Gray, J., G.S. Yeo, J.J. Cox, J. Morton, A.L. Adlam, J.M. Keogh, J.A. Yanovski, A. El Glarabwy, J.C. Horne, C. Yang, et al. 2005. Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. Diabetes. 55:3366–3371.  http://dx.doi.org/10.2337/db06-0550

Hartmann, M., R. Heumann, and V. Lessmann. 2001. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J. 20:5887–5897.  http://dx.doi.org/10.1093/emboj/20.21.5887

Haubensak, W., F. Narz, R. Heumann, and V. Lessmann. 1998. BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. J. Cell Biol. 111:1483–1493.

Heyden, A., M.C. Ionescu, S. Romorini, B. Kracht, V. Giugliori, P. Calabresi, C. Seidenbecher, F. Angenstein, and E.D. Gundelfinger. 2011. Hippocampal enlargement in Bassoon-mutant mice is associated with enhanced neurogenesis, reduced apoptosis, and abnormal BDNF levels. Cell Tissue Res. 344:11–26.  http://dx.doi.org/10.1007/s00441-011-1233-3

Hofer, M., S.R. Pagliusi, A. Hohn, J. Leibrock, and Y.A. Barde. 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J. 9:2459–2464.

Isackson, P.J., M.M. Huntsman, K.D. Murray, and C.M. Gall. 1991. BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: Temporal patterns of induction distinct from NGF. Neuropharmacology 30:937–948.  http://dx.doi.org/10.1016/0028-3908(91)90115-3

Jaksiewicz, S.K., H.B. Nasser, M.J. Strong, A.J. McCartney, A.S. Perez, N. Rakies, C.J. Carruthers, and M.A. Sutton. 2010. Local presynaptic activity gates homeostatic changes in presynaptic function driven by dendritic BDNF synthesis. Neuron. 68:1143–1158.  http://dx.doi.org/10.1016/j.neuron.2009.10.034

Jones, K.R., I. Farinas, C. Backus, and L.F. Reichardt. 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell. 76:989–999.  http://dx.doi.org/10.1016/0092-8674(94)90377-8

Karey, K.P., and D.A. Siraskar. 1989. Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for western blot analysis. Anal. Biochem. 178:255–259.  http://dx.doi.org/10.1016/0003-9861(89)90634-9

Kennedy, M.J., and M.D. Ehlers. 2011. Mechanisms and function of dendritic exocytosis. Neuron. 69:856–875.  http://dx.doi.org/10.1016/j.neuron.2011.02.032

Kerr, A.M., and P. Jonas. 2008. The two sides of hippocampal mossy fiber plasticity. Nature. 57:7–7.  http://dx.doi.org/10.1038/57011201

Kojima, M., N. Takei, T. Numakawa, Y. Ishikawa, S. Suzuki, T. Matsumoto, R. Katoh-Semba, H. Nawa, and H. Hatanaka. 2001. Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest its activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J. Neurosci. Res. 64:1–10.  http://dx.doi.org/10.1002/jnr.1001080

Kolbeck, R., I. Bartke, W. Eberle, and Y.A. Barde. 1999. Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice. J. Neurosci. 19:390–398.  http://dx.doi.org/10.1523/JNEUROSCI.19-02-0390.1999

Korte, M., P. Carroll, E. Wolf, G. Brem, H. Thoenen, and T. Bonhoeffer. 1995. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. USA. 92:8856–8860.  http://dx.doi.org/10.1073/pnas.92.19.8856

Presynaptic localization of BDNF • Dieni et al. 787
Koshimizu, H., K. Kiyosue, T. Hara, S. Hazama, S. Suzuki, K. Uegaki, G. Nagappan, E. Zaitsev, T. Hirokawa, Y. Tatsu, et al. 2009. Multiple functions of precursor BDNF to CNS neurons: Negative regulation of neurite growth, spine formation and cell survival. Mol. Brain. 2:27. http://dx.doi.org/10.1186/1756-6606-2-27

Lang, S.B., V. Stein, T. Bonhoeffer, and C. Lohmann. 2007. Endogenous brain-derived neurotrophic factor triggers fast calcium transients at synapses in developing dendrites. J. Neurosci. 27:1097–1105. http://dx.doi.org/10.1523/JNEUROSCI.3590-06.2007

Lavebratt, C., A. Trifunovski, A.S. Persson, F.H. Wang, T. Klasen, I. Ohman, A. Josephsson, J. Olson, C. Spinner, and M. Schalling. 2006. Carbacholamine protects against megenepchla and abnormal expression of BDNF and Nogo signaling components in the mepch/mepch mouse. Neurobiol. Dis. 24:374–383. http://dx.doi.org/10.1016/j.nbd.2006.07.018

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. Nature. 341:149–152. http://dx.doi.org/10.1038/341149a0

Li, Y., G. Calfa, T. Inoue, M.D. Amaral, and L. Pozzo-Miller. 2010. Activity-dependent release of endogenous BDNF from mossy fibers evokes a TRPC3 current and Ca2+ elevations in CA3 pyramidal neurons. J. Neurophysiol. 103:2846–2856. http://dx.doi.org/10.1152/jn.01140.2009

Link, W., U. Konietzko, G. Kaussmenn, M. Krug, B. Schwanke, U. Frey, and D. Kuhl. 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc. Natl. Acad. Sci. USA. 92:5734–5738. http://dx.doi.org/10.1073/pnas.92.13.5734

Lohivuori, V., A. Vicario, M. Uutela, T. Rantamaki, L.M. Lohivuori, E. Castrén, E. Tongiorgi, K.E. Akerman, and M.L. Castrén. 2011. BDNF and TrkB in neuronal differentiation of Fmr1-knockout mouse. J. Neurosci. 31:469–480. http://dx.doi.org/10.1523/JNEUROSCI.0101-10.2010

Luo, X.G., R.A. Rush, and X.F. Zhou. 2001. Ultrastructural localization of brain-derived neurotrophic factor in rat primary sensory neurons. Neurosci. Res. 39:377–384. http://dx.doi.org/10.1016/S0161-6420(00)00238-8

Lyford, G.L., K. Yamagata, W.E. Kaufmann, C.A. Barnes, L.K. Sanders, N.G. Copeland, D.J. Gilbert, N.A. Jenkis, A.A. Lanahan, and P.F. Worley. 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskelaton-associated protein that is enriched in neuronal dendrites. Neuron. 14:433–445. http://dx.doi.org/10.1016/0896-6273(95)90299-6

Matsuda, N., H. Lu, Y. Fukuta, J. Noritahe, H. Gao, S. Mukjerhee, T. Nemoto, M. Fukuta, and M.M. Poo. 2009. Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J. Neurosci. 29:1485–1498. http://dx.doi.org/10.1523/JNEUROSCI.1219-09.2009

Matsumoto, T., S. Rauskolb, M. Polack, J. Kloese, R. Kolbeck, M. Korte, and Y.A. Barde. 2008. Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat. Neurosci. 11:131–133. http://dx.doi.org/10.1038/nn2038

Mellman, I., and W.J. Nelson. 2008. Coordinated mRNA and protein expression of proBDNF and pro-TrkB in developing neurons. Nat. Neurosci. 11:131–133. http://dx.doi.org/10.1038/nn2038

Miller, S., M. Yasuda, J.K. Coats, Y. Jones, M.E. Martone, and M. Mayford. 2008. Coordinated protein sorting, targeting and transport of BDNF and its Trk receptors. Nat. Rev. Neurosci. 9:3545–3550. http://dx.doi.org/10.1152/physrev.00041.2004

Nicoll, R.A., and D. Schmitz. 2005. Synaptic plasticity at hippocampal mossy fiber synapses. Nat. Rev. Neurosci. 6:863–876. http://dx.doi.org/10.1038/nrn1786

Nikolaoupolou, V., H. Lickert, J.M. Frade, C. Rencurel, P. Giaillonde, L. Zhang, M. Biehl, and Y.A. Barde. 2010. Neurotrophin receptors TrkA and TrkB cause neuronal death whereas TrkD does not. Nature. 467:59–64. http://dx.doi.org/10.1038/nature09336

Patterson, S.L., T. Abel, T.A. Deuel, K.C. Martin, J.C. Rose, and E.R. Kandel. 2005. Synaptic plasticity at hippocampal mossy fiber synapses as revealed by high-pressure freezing. J. Neurosci. 30:1739–1749. http://dx.doi.org/10.1523/JNEUROSCI.3100-10.2010

Rakic, J.L., C.J. Sandowal, F. Bermudez-Rattoni, and A. Routtenberg. 2007. Remodeling of hippocampal mossy fibers is selectively induced seven days after the acquisition of a spatial but not a cued reference memory task. Learn. Mem. 14:416–421. http://dx.doi.org/10.1101/lm.516507