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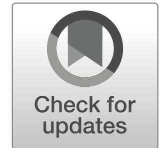
REVIEW



Journey of brain-derived neurotrophic factor: from intracellular trafficking to secretion

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Abstract
Brain-derived neurotrophic factor (BDNF) is known to control a wide variety of brain functions, ranging from memory formation to food intake. However, since the BDNF levels are extremely low in the nervous system, the dynamics in neurons from intracellular trafficking to secretion is absolutely complicated; the understanding is not fully promoted. We here review the findings of those critical mechanisms from intracellular trafficking to the secretion of BDNF. Furthermore, to solve this issue, technological advances for the detection, measurement, and imaging of this growth factor are essential. We believe that this review helps the study of these complex but critical mechanisms of BDNF.

Keywords Brain-derived neurotrophic factor · Neurotrophins · Synaptic transmission · BDNF pro-peptide · Ca2+-dependent activator protein for secretion 2 · Exocytosis

The neurotrophin family

Neurotrophins (NTs) historically emerged as a family of polypeptides for promoting neuronal survival and differentiation and have recently been studied as a class of modulators for synaptic transmission and plasticity (Bibel and Barde 2000; Park et al. 2008). In the 1950s, the group of Levi-Montalcini and Hamburger found that when a mouse sarcoma tumor was implanted near the spinal cord of the developing chicken the neurons exhibited neurite outgrowth (Levi-Montalcini and Hamburger 1953). The extensive efforts after the finding led to the successful identification of a growth factor namely nerve growth factor (NGF) (Bocchini and Angeletti 1969; Cohen 1960; Cohen and Levi-Montalcini 1956). Their discovery of NGF played a pioneering role in the studies of developmental neurobiology, which raised a new hypothesis that target-derived secreted proteins control neurite growth and neuronal survival.

Over 20 years after the discovery of NGF, Barde et al. (1982) isolated a neuronal survival-eliciting factor from the pig brain, named brain-derived neurotrophic factor (BDNF) (Barde et al. 1982). In 1989, the primary structure of BDNF and its expression in the brain was identified (Leibrock et al. 1989). Interestingly, it was demonstrated that BDNF is highly homologous to NGF at the level of the amino acid sequence, and using a technology of polymerase chain reaction (PCR), neurotrophin-3 (NT-3) (Maisonpierre et al. 1990), and neurotrophin-4/5 (NT-4/5) (Ip et al. 1992), whose amino acid sequence is very homologous to NGF and BDNF, was identified one after another. These four NTs and their genes showed marked homology to each other in terms of sequence and structure (Lessmann et al. 2003). These discoveries of NTs family have provided novel insights into the formation of neuronal communication during the development of the nervous system and into synaptic plasticity, memory, and learning in the adult brain. Interestingly, it was demonstrated that, for the formation of specific neuronal communications spatially and temporally, one or more NTs are required. Furthermore, there are long- and short-term actions of NTs. The long-term NT action depends on gene regulation, whereas

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for short-term effects, including chemotrophic effects on developing neurons and synaptic transmissions, the activation of cytoplasmic effectors by NTs was shown to be required.

NT receptors and their signaling

There have been prolonged efforts to identify the receptors of NTs. Two distinct classes of NT receptors have been discovered. The first receptor, the p75 NT receptor ($p75^{NTR}$), is a member of the tumor necrosis factor (TNF) receptor family (Chao 2003). $p75^{NTR}$ was initially discovered as a low-affinity NGF receptor but was found to bind BDNF, NT-3, and NT-4/5 with a similar affinity (for review, (Chao 2003)). The extracellular domain of $p75^{NTR}$ has cysteine-rich motifs. The cytoplasmic domain of $p75^{NTR}$ includes a "death" domain, similar to that of the TNF receptor family (He and Garcia 2004; Liepinsh et al. 1997). Interestingly, while $p75^{NTR}$ does not contain a catalytic kinase motif, it interacts with many proteins that transmit signals for controlling neuronal survival and differentiation (Chao 2003; Hempstead 2014). The $p75^{NTR}$ receptor activates three major signaling pathways (for review, (Hempstead 2002)) (also see articles by Korte et al., Brigadski et al., and Mobley et al., in this special issue). First, NF-kappa B activation leads to the transcription of multiple genes, some of which promote neuronal survival. Second, activation of the Jun kinase pathway similarly controls the activation of several genes, some of which promote neuronal apoptosis. Third, ligand engagement of $p75^{NTR}$, through the regulation of Rho activity, controls the motility of growth cone.

The second class of NT receptors is the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) (for review, (Reichardt 2006)). Each NT binds to Trk receptor in a specific manner: NGF binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. The Trk receptors have a trans-membrane region that spans the plasma membrane and a cytoplasmic domain that has tyrosine kinase activity. Binding of a NT to a specific Trk receptor activates its tyrosine kinase, leading to the activation of phosphatidylinositol 3-kinase (PI_3K), mitogen-activated protein kinase (MAPK), and phospholipase C-γ (PLC-γ) pathways. Notably, it was demonstrated that mutation of TrkB receptors at the PLC-γ docking site, but not the Shc site, impaired hippocampal long-term potentiation (LTP) (Minichiello et al. 2002). Many additional adaptors for $p75^{NTR}$ and Trk receptors have been reported. Information about the two types of NT receptors and their signaling mechanisms has been extensively reviewed by others (for review, (Chao 2003, Hempstead 2002, Ibanez and Simi 2012, Reichardt 2006)) and will not be further discussed in this review.

A critical mechanism for NT action: secretion and intracellular transportation of NT

NTs are initially synthesized as precursors composed of pre-pro-neurotrophins. The precursor proteins are structurally composed of a signal sequence, a pro-domain, and the mature domain. To produce the mature and bioactive form, the signal sequence and the next N-terminal fragment or the "pro-domain" are proteolytically processed by intracellular and/or extracellular proteolytic enzymes (e.g., furin, pro-hormone convertase, and plasmin) (Lee et al. 2001; Lu et al. 2005; Pang and Lu 2004; Seidah et al. 1996). Several reports explored the intracellular mechanism of NT processing. The N-terminal pro-domain is proteolytically processed in either *trans*-Golgi by furin or secretory granules by intracellular proteinases to generate a mature form of neurotrophins while in non-neuronal cells, neurotrophins are constitutively secreted (Seidah et al. 1996).

It was previously reported that the BDNF secretion is controlled by both constitutive and activity-regulated secretion pathways in cultured neurons (Mowla et al. 1999). The activity-dependent secretion of BDNF is controlled in a neuronal activity- and Ca^{2+}-dependent manner (for review, (Lessmann and Brigadski 2009)) (also see Brigadski and Lessmann in this special issue). There is a large study indicating that the activity-dependent secretion of BDNF is a critical mechanistic step in controlling synaptic transmission and long-term synaptic plasticity (Park and Poo 2013). Furthermore, the exonic Val66Met polymorphism, in which an amino acid residue substitution alters valine to methionine at codon 66 within the BDNF pro-domain, has been reported to be a functional single-nucleotide polymorphism (SNP) that results in deficient BDNF translocation and secretion as well as impaired episodic memory in human (Egan et al. 2003). This article first showed that the BDNF itself and its mechanism of secretion and translocation is important for the brain function in human. We next review the articles that study the secretion and translocation of BDNF.

Biogenesis and transport of BDNF-containing vesicles

Like other growth factors, BDNF protein is produced as a precursor polypeptide, proBDNF (32 kDa), in the rough endoplasmic reticulum (rER), and is processed to mature BDNF ($\sim$ 14 kDa) during the sorting pathway from the rER-Golgi (processed by furin) to dense core vesicle (DCV) like secretory vesicles (by proconvertases), and extracellularly by plasmin and metalloproteinases MMP3 and MMP7 (for reviews, see Lessmann and Brigadski (2009), Song et al. (2017). BDNF synthesized in the rER is transported into the *trans*-Golgi network (TGN) and is packed and stored in DCVs.

Granin (chromogranin and secretogranin), which plays a potential regulatory molecule for the packing of BDNF in DCVs, is a highly acidic, Ca^{2+}-binding, and prone-to-aggregate protein. Several members of this family are abundantly localized in DCVs and are possibly involved in the sorting and packing of peptide precursors into DCVs (Bartolomucci et al. 2011). Secretogranin II (SCG2/chromogranin C) of the granin family is localized in BDNF vesicles in hippocampal neurons.

There are two sorting receptors for BDNF to the regulatory secretion pathway: a VPS10 domain family protein sortilin (SORT1), which interacts with the pro-domain of BDNF, and carboxypeptidase E (CPE), which interacts with BDNF in the TGN. Sortilin (SORT1), a member of the Vps10p sorting receptor family specifically interacts with the pro-domain to sort BDNF to the regulated secretory pathway (Chen et al. 2005). The lipid-raft-associated sorting receptor CPE likely sorts proBDNF into regulated pathway vesicles for activity-dependent secretion in cortical and hippocampal neurons (Lou et al. 2005). CPE also binds dynactin that recruits kinesins kinesin family member 1A (KIF1A and kinesin family member 1A (KIF3A) (plus-end microtubule-based motors) or dynein (a minus-end-directed motor) for driving bidirectional (anterograde and retrograde) transport of BDNF vesicle to maintain vesicle homeostasis and secretion in hippocampal neurons (Park et al. 2008).

Quantitative measurement and immunocytochemistry of endogenous BDNF

While many aspects of the BDNF role in the brain have been understood, the subcellular localization and concentration of this releasable protein should be determined carefully. One important reason is that endogenous levels of BDNF are very low. Therefore, to understand the mechanism underlying spatial and temporal actions of BDNF, the methodology of the quantitative measurement, immunocytochemistry, and imaging of BDNF should be convincing.

For quantification of endogenous BDNF, a two-site enzyme-linked immunosorbent assay (ELISA) is used, although there are some methodological issues to apply this method to human samples. One problem is that the BDNF levels in serum, plasma, and whole blood are divergent (see Karege et al. 2005). A method to obtain reliable measurements of human serum BDNF has been proposed by comparing six commercially available kits (Polacchini et al. 2015). Several factors including rodent strain, extraction buffer used, and dilution of the sample are pointed out (Angelucci et al. 2003; Angelucci et al. 2000).

To investigate the intracellular localization of endogenous BDNF, not only highly sensitive antibody but control experiment, for instance, a comparison of the immunoreactive signal between wild-type mice and its littermate lacking BDNF gene is required. Recently, two reports clearly showed the intracellular localization of endogenous BDNF clearly. Dieni et al. (2012) demonstrated a beautiful immunocytochemical localization of endogenous BDNF in the adult mouse hippocampus (Dieni et al. 2012). They carefully investigated the distribution of BDNF at both light microscopic and ultrastructural levels using three lines of transgenic animals. As a negative control, they performed their study using a line conditionally lacking BDNF gene in adult neurons (Rauskolb et al. 2010). In the report of Dieni et al. (2012), they suggested an anterograde mode of action of BDNF, which contrasts with the long-established retrograde model of NGF in the peripheral nervous system.

For the detection of endogenous BDNF, molecular tagging is also useful. In the study using *Bdnf*-hemagglutinin (HA) knock-in mouse line, Yang et al. (2009) showed that proBDNF was releasable at the prenatal stage and still detectable in adulthood (Yang et al. 2009). Interestingly, they also observed highest levels of proBDNF and neurotrophin receptor p75 at the perinatal stage and their expression levels declined in adulthood (Yang, Siao, Nagappan, Marinic, Jing, McGrath, Chen, Mark, Tessarollo, Lee, Lu and Hempstead, 2009), suggesting that the biological action of proBDNF, through the activation of p75, is distinct form that of BDNF (Barker 2009). Recently, Harward et al. (2016) performed electron microscopy to examine BDNF localization in a mouse line in which a C-terminal HA epitope tag was added to the Bdnf-coding sequence (Bdnf-HA) (Yang, Siao, Nagappan, Marinic, Jing, McGrath, Chen, Mark, Tessarollo, Lee, Lu and Hempstead, 2009). An immune-electron microscopy study using a highly sensitive antibody against the HA-tag showed the presence of endogenous BDNF not only in axons but also in dendrites and spines of CA1 pyramidal cells of these mice (Harward et al. 2016).

Using specificity controls, Dieni et al. (2012) showed that antibodies reacting either with BDNF or its pro-peptide (BDNF pro-peptide, ~ 17 kDa) both stained large dense core vesicles in excitatory presynaptic terminals of the adult mouse hippocampus, indicating that the BDNF and its pro-peptide are co-stored in and is able to be co-secreted from the same BDNF vesicles (Dieni, Matsumoto, Dekkers, Rauskolb, Ionescu, Deogracias, Gundelfinger, Kojima, Nestel, Frotscher and Barde, 2012). In line with this finding, Uegaki et al. (2017) showed that the BDNF pro-peptide binds to mature BDNF with high affinity on a BIAcore sensor chip (Uegaki et al. 2017). Furthermore, they showed that this interaction was more enhanced at acidic pH than at neutral pH (Uegaki, Kumanogoh, Mizui, Hirokawa, Ishikawa and Kojima, 2017), suggesting that BDNF and its pro-peptide are present together in intracellular compartments such as trafficking vesicles.

Table 1 Tools for monitoring BDNF release

Tools	Features	References
BDNF antibody	Immunoassay using poly- or mono-clonal antibodies specific to BDNF or proBDNF *[pros & cons] reliable and high sensitivity but non-real-time measurement system	Katoh-Semba et al. (1999), Kolbeck et al. (1999), Koshimizu et al. (2009)
BDNF-GFP/EGFP	Expression of recombinant BDNF fused to GFP or enhanced GFP (EGFP) [pros & cons] cellular imaging of vesicular transport and release but exogenous or ectopic expression	Egan et al. (2003), Hartmann et al. (2001), Kohara et al. (2001), Kojima et al. (2001), Egan (2003), Adachi et al. (2005), Brigadski et al. (2005), Eckenstaler et al. (2016), Kuczewski et al. (2008), Matsuda et al. (2009), Eckenstaler et al. (2016)
BDNF-pHluorin	Expression of recombinant BDNF fused to pHluorin (pH-sensitive GFP derivative) [pros & cons] real-time imaging of release kinetics but exogenous or ectopic expression	Dean et al. (2009), Matsuda (2009), Shinoda et al. (2011), Harward (2016), Matsuda (2009), Shimojo et al. (2015), Shinoda et al. (2011), Harward et al. (2016)
BDNF-PAGFP	Expression of recombinant BDNF fused to PAGFP (photoactivatable GFP) [pros & cons] photoactivatable, pulse-chase imaging but exogenous or ectopic expression	Dean et al. (2012)
BDNF-Myc	Expression of recombinant BDNF fused to Myc tag [pros & cons] reliable and high sensitivity but non-real-time measurement system	Dean et al. (2012)
Bescell (BDNF sensor cell)	Cell-based fluorescent indicator using the expression of a chimeric receptor containing the BDNF binding domain and FRET (Förster resonance energy transfer) domain. [pros & cons] cellular imaging of released BDNF but exogenous or ectopic expression	Nakajima et al. (2008)
FRET-based sensor for BDNF-TrkB signaling	FRET between the EGFP fused with the C-terminal of TrkB and the SH2 domain of PLC-γ1 flanked by two mRFPs. [pros & cons] analyzing intracellular signaling activated by BDNF but an exogenous or ectopic expression of TrkB	Harward et al. (2016)
BDNF-HA knock-in mouse	Mouse *Bdnf* sequence with a C-terminal hemagglutinin (HA) epitope tag (Bdnf-HA) [pros & cons] analyzing the endogenous expression of BDNF in vivo but non-real-time measurement system	Yang et al. (2009)
BDNF-GFP knock-in (KiBE) mouse	The mouse *Bdnf* exon 9 sequence is fused at its C terminus in the frame to the EGFP coding sequence. [pros & cons] imaging of in vivo trafficking and release but probably lower sensitivity than exogenous overexpression	Leschik et al. (2019)

*Possible pros and cons for each technique are also indicated

Biochemical detection and imaging of BDNF release

How is BDNF secreted from neurons? To address this question, a variety of BDNF release assay and imaging techniques has been developed. To measure the released BDNF levels, the biochemical method is technically solid. In particular, specific antibody and sensitive immunoassay methods were recently useful. The release from cells into culture media can conventionally be measured for BDNF (Anastasia et al. 2013, Katoh-Semba et al. 1999, Kolbeck et al. 1999, Matsumoto et al. 2008, Rauskolb, Zagrebelsky, Dreznjak, Deogracias, Matsumoto, Wiese, Erne, Sendtner, Schaeren-Wiemers, Korte and Barde, 2010), its pro-peptide (BDNF pro-peptide) (Dieni, Matsumoto, Dekkers, Rauskolb, Ionescu, Deogracias, Gundelfinger, Kojima, Nestel, Frotscher and Barde, 2012, Mizui et al. 2019, Mizui et al. 2015), and proBDNF (Yang, Siao, Nagappan, Marinic, Jing, McGrath, Chen, Mark, Tessarollo, Lee, Lu and Hempstead, 2009). To detect the endogenous BDNF pro-peptide in Western blotting, membrane fixation with glutaraldehyde was technically effective (Dieni, Matsumoto, Dekkers, Rauskolb, Ionescu, Deogracias, Gundelfinger, Kojima, Nestel, Frotscher and Barde, 2012, Matsumoto et al. 2008, Mizui et al. 2019, Mizui et al. 2015).

The cell imaging methods utilizing the expression of recombinant BDNF fused to fluorescent proteins such as green fluorescent protein (GFP) and its derivatives are a most used approach (Table 1). The luminal side of vesicles is acidic, so that fluorescence of pH-sensitive GFP derivatives such as pHluorin extinct inside of the vesicles, and is emitted upon exocytosis due to being exposed to the neutral solution via its release out of cells. If fusion pore is already open (i.e., the

intra-vesicular pH becomes neutral), pHluorin fluorescence is unquenched even in the absence of release. For the purpose to measure small amounts of intrinsic BDNF, imaging animal models carrying modified BDNF have also been developed (Table 1).

By monitoring fluorescent changes of BDNF-GFP before and after exocytosis, it was shown that BDNF is activity dependently released at synaptic sites of cultured rat hippocampal neurons in response to depolarization induced by application of high KCl (Kojima et al. 2001), of cultured mouse cortical neurons in response to picrotoxin (Kohara et al. 2001), and of cultured rat hippocampal neurons in response to either high K^+/glutamate or electrical stimulation (depolarization and tetanus) (Hartmann et al. 2001). Interestingly, BDNF-GFP expressed in axons of cultured mouse cortical neurons was dominantly transported in an anterograde direction, whereas that in dendrites mostly stayed or fluctuated back and forth within a short distance (Adachi et al. 2005). Notably, the Val66Met polymorphism affects the activity-dependent release of BDNF. Met variants showed lower activity-dependent release from cultured rat hippocampal neurons, while its constitutive release is unchanged (Egan et al. 2003).

Imaging of dendritic BDNF release

Hippocampal neurons in vivo are normally exposed to tonic activities ranging from 1 to 20 Hz and brief high-frequency (50 Hz) bursting activities (Bland 1986). Under these conditions, BDNF-GFP heterologously overexpressed in cultured rat hippocampal neurons is secreted predominantly from the dendrite and acts on presynaptic terminals as a retrograde signal or on the postsynaptic cell as an autocrine factor (see Brigadski and Lessmann in this special issue). The dendritic release at postsynaptic and extra-synaptic sites is elicited by Ca^{2+} influx via ionotropic glutamate receptor (iGluR) or differential voltage current conveyor (DVCC) in rat hippocampal neurons (Hartmann et al. 2001) (also see Brigadski and Lessmann 2020 in this special issue). The kinetics of depolarization (50 mM KCl)-induced postsynaptic BDNF-EGFP release was analyzed using a similar culture system (time constant $\tau = 307 \pm 78$ s; half decay time $t_{1/2} = 288 \pm 4$ s) (Brigadski et al. 2005). Interestingly, BDNF is a sticky protein and the release time became faster ($\tau = 81 \pm 22$ s; $t_{1/2} = 52 \pm 14$ s) after treatment with monensin (to neutralize intra-vesicular pH) prior to depolarization, suggesting a possible effect of pH-dependent peptide condensation in the acidic luminal condition on vesicular exocytosis. These authors also showed that BDNF-EGFP showed slower release time course than three other EGFP-fused NTs (NT-3, NGF, and NT-4) and that BDNF and NT-3 are more efficiently sorted to the regulated dendritic release, whereas NGF and NT-4 are more efficiently sorted to constitutive release (Brigadski et al. 2005). The activity-dependent dendritic release of BDNF-GFP was also shown to be triggered by spontaneous back-propagating action potentials (bAPs) that induce Ca^{2+} influx through voltage-dependent calcium channels (VDCCs) (Kuczewski et al. 2008). In cultured rat hippocampal neurons transfected with BDNF-pHluorin, 10-Hz stimulation (1 min, 300 pulses) triggers full-collapse vesicle fusion and substantial BDNF release at the dendrite presumably by triggering bAPs and Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCCs) (Matsuda et al. 2009). In CA1 pyramidal neurons of acute rat hippocampal slices, a theta burst of postsynaptic action potentials (APs) elicited BDNF-dependent postsynaptic timing-dependent long-term potentiation (t-LTP, a physiologically relevant type of synaptic plasticity that results from the repeated sequential firing of APs in pre- and postsynaptic neurons) that relied on postsynaptic BDNF release and its autocrine action at the postsynaptic CA1 neurons (Edelmann et al. 2015). The authors noted that although Dieni et al. (2012) does not significantly detect BDNF in dendritic compartments of CA1 neurons by immunochemistry (Dieni et al. 2012) (see the beginning of Chapter 8), endogenous levels of BDNF in CA1 dendrites—while sufficient to affect t-LTP—might be undetectable levels by antibody detection. In cultured rat hippocampal slices, biolistically transfected with BDNF-pHluorin, the rapid release is evoked N-methyl-D-aspartate receptor (NMDAR)-calcium/calmodulin-dependent protein kinase II (CaMKII) dependently from dendritic spines stimulated with uncaged glutamate, which activates an autocrine signaling system (Harward et al. 2016). In acute hippocampal slices of BDNF-GFP knock-in (KiBE) mice, 70% of BDNF-GFP vesicles are localized in dendrites and their release shows about 30-s delay after depolarization and continues for more than 100 s thereafter (Leschik et al. 2019).

Imaging of axonal BDNF release

Dieni et al. (2012) provided the evidence showing by using specific antibodies (see Chapter 5) that both BDNF and its pro-peptide are released from axons in an anterograde fashion rather than from postsynaptic dendrites/spines in the adult hippocampus (Dieni et al. 2012). They noted that the use of in vitro cultured neurons transfected with BDNF constructs probably grow in very different conditions compared with in vivo ones and express significantly higher levels of BDNF detectable in the adult hippocampus, which may cause ectopic localization of exogenous BDNF. Recently, Leschick et al. (2019) developed a knock-in mouse line which Bdnf exon 9 sequence was fused with constitutively fluorescent EGFP protein (KiBE) and visualized that the BDNF-EGFP-containing vesicles in pyramidal cell dendrites of hippocampal slices prepared from the imaging mouse model KiBE

(Leschik et al. 2019). This study is a kind of challenging because, as described earlier, BDNF levels contained in neurons and tissues are not plenty, and the content depends on cell type, states and development, and circadian rhythms analyzed (Hamatake et al. 2011). Given such difficulty, the development of technology for precisely visualizing the localization and release of BDNF should be important to address the remaining yet important questions, including the relationship between axonal-presynaptic release and dendritic-postsynaptic release, in the field of BDNF biology.

Immunohistochemical studies demonstrated that BDNF protein derived from anterograde axonal transport was detected in axonal processes and preferentially stored in terminals within the innervation targets of rat brain (Conner et al. 1997). BDNF was also shown to be anterogradely transported in catecholaminergic axon terminals projected to rat lateral septum (Fawcett et al. 2000). Moreover, heterogeneously expressed BDNF-GFP moved in axons of cultured mouse cortical neurons in the anterograde direction, though some moved retrogradely, and transferred to postsynaptic neurons in an activity-dependent manner (Kohara et al. 2001). Immuno-electron microscopic study demonstrated that BDNF- and pro-domain-containing DCV-like vesicles are abundantly localized to excitatory presynaptic terminals, such as mossy fiber terminals, of the adult mouse hippocampus, indicating an anterograde mode of BDNF action (Dieni et al. 2012).

By using in situ ELISA technique, it was shown that 100-Hz tetanus and theta burst stimulation rather than low-frequency stimulation triggered the more efficient release of native BDNF from newborn rat hippocampal neurons by either Ca^{2+} influx via the N-type VDCC or Ca^{2+} release from the internal store (Balkowiec and Katz 2002). In CA3-CA1 synapses of hippocampal slices, presynaptic BDNF release from the Shaffer collaterals of CA3 neurons is physiologically required to recruit the presynaptic (200 Hz- or theta burst-induced long-term potentiation, LTP), but not postsynaptic (50 Hz-induced LTP) module of plasticity (Zakharenko et al. 2003). Presynaptically released BDNF was suggested to act on TrkB at the presynaptic terminals in an autocrine mode or at postsynaptic spines in an anterograde mode. In cultured rat hippocampal neurons transfected with BDNF-pHluorin, the activity-dependent axonal release was also reported (Matsuda et al. 2009). A brief spiking activity (10 Hz, 300 pules) triggers a transient fusion pore opening followed by immediate retrieval of vesicles, resulting in very little partial BDNF release at the axon. However, full-vesicular fusion with BDNF secretion could occur at the axon when the neuron was stimulated by prolonged high-frequency activity (required sustained high-frequency tonic or bursting activities for a few minutes) (Matsuda et al. 2009).

Regulatory molecules underlying the exocytosis of BDNF vesicles (Fig. 1)

BDNF is activity dependently released via exocytosis of DCV-like secretory vesicles. In the same way as exocytosis of synaptic vesicles (SVs), BDNF is released to the outside of the cells through fusion pore that is formed by membrane fusion between the secretory vesicles and the plasma membrane. The basic machineries for SV exocytosis, including the soluble NSF attachment protein receptor (SNARE) proteins and associated regulatory molecules, are well studied and are thought to be similarly utilized in exocytosis of BDNF vesicles. Although in this way the same regulatory molecules and some different isoforms seem to be involved in exocytosis of BDNF vesicles, the detailed molecular machineries that precisely tune the release of BDNF which is so effective even in very small amounts are yet unclear.

Three SNARE proteins localized to vesicle membrane (synaptobrevin/VAMP) and target membrane (syntaxin and SNAP25) are the main machinery for vesicle fusion at the active zone or the release site membrane and consist of multiple isoforms of each member. Their isoforms synaptobrevin/VAMP2 (Syb2) and synaptosomal-associated protein (SNAP25) mediate the vesicular release of BDNF-pHluorin in axons and dendrites of cortical neurons (Shimojo, Courchet, Pieraut, Torabi-Rander, Sando, Polleux and Maximov, 2015). Remarkably, axonal secretion of BDNF is also regulated by SNAP47, an atypical member of the SNAP family (Shimojo, Courchet, Pieraut, Torabi-Rander, Sando, Polleux and Maximov, 2015). The interaction of mammalian uncoordinated-13 (Munc13) and Rab3-interacting molecule 1 (RIM1) is thought to target Rab3-containing SVs to the active zone. The N-terminal domain of RIM1/2 interacts with both Rab3A anchoring to DCV membrane and Munc13 binding to PIP_2 distributed within the release site membrane. RIM1/2 is an essential Ras-related protein 3A (Rab3A) effectors and is essential for DCV fusion for the regulated release of neuropeptides in hippocampal neurons and organize DCV fusion by positioning/activating acronym for Munc13 and recruiting DCVs through Rab3 interactions (Persoon et al. 2019). Although the release of NPY-pHluorin was analyzed in this study, the release of BDNF seems to be similarly conducted by utilizing this kind of machinery.

In addition to the vesicle targeting and fusion machineries, some positive and negative regulators for the exocytosis pathway of BDNF vesicles have been identified, by which BDNF release is thought to be finely tuned. Synaptotagmin (Syt) is an integral membrane protein on SVs and acts as a Ca^{2+} sensor to trigger the SNARE-mediated vesicle fusion. Its isoform

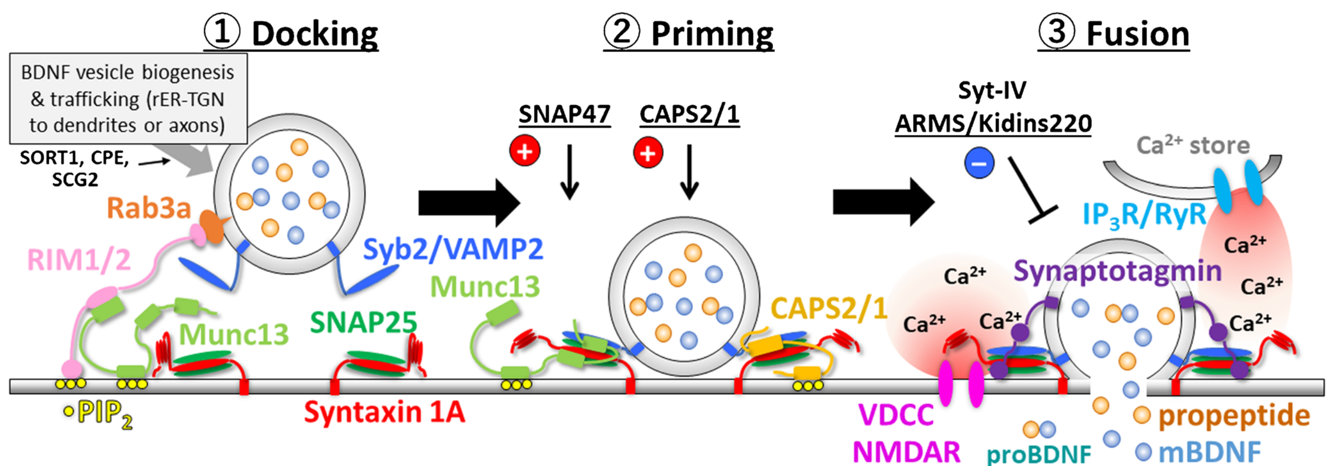

Fig. 1 Molecular components underlying the exocytosis of DCV containing BDNF and pro-peptide. The exocytosis of DCV is implemented by SNARE complex (syntaxin 1A, SNAP25, and synaptobrevin-2 [Syb2]/VAMP2) and consists of three steps: docking, priming, and membrane fusion. ① Docking: DCV is recruited to the release site on the plasma membrane by several proteins—e.g., Rab3a, RIM1/2, and Munc13. proBDNF is included in and transported by the vesicle prior to the docking step—i.e., through the rER-TGN trafficking steps during which a fraction of proBDNF is luminally processed into pro-peptide and mBDNF. ② Priming: Munc13, in turn, plays a key role in stabilizing SNARE component(s), which renders DCV ready to be released. CAPS2 (and/or CAPS1, presumably) is also involved in the priming step in a different manner from Munc13. Specific phospholipids of the plasma membrane, such as PIP_2, are likely to support the molecular functions of these priming proteins. ③ Fusion: following the Ca^{2+} influx form outside the cell via VDCC and/or NMDAR, as well as the Ca^{2+} release from internal stores via IP_3 receptor (IP_3R) or ryanodine receptor (RyR), a Ca^{2+} sensor synaptotagmin (Syt) on the DCV membrane controls the timing of vesicle release. Consequently, SNARE complex executes DCV exocytosis by bringing together the plasma membrane and DCV membrane mechanically, resulting in spilling out BDNF, pro-peptide, or proBDNF enclosed in DCV. Released proBDNF is extracellularly processed by specific proteases. Release of BDNF is controlled positively by CAPS2/1 (by interacting with syntaxin) and SNAP47 (by binding to Syb2/VAMP2 and SNAP25) and negatively by Syt-IV and ARMS/Kidins220

syaptotagmin-IV (Syt-IV) localizes to BDNF vesicles and can interact with SNARE proteins, while it fails to bind to phosphatidylinositol bisphosphate$_2$ (PIP_2)-containing plasma membrane in response to Ca^{2+}, resulting in the inhibition of dendritic and axonal BDNF release BDNF release (Dean et al. 2009). Therefore, Syt-IV acts as a negative regulator for exocytosis of BDNF vesicles and modulates synaptic function and long-term potentiation (Dean et al. 2009; Dean et al. 2012). Ankyrin repeat-rich membrane spanning protein ARMS/Kindins220 is a scaffold protein that interacts with multiple proteins including Trk and p75 receptors and negatively regulates BDNF release by interacting with Syt-IV (López-Benito et al. 2018).

The Ca^{2+}-dependent activator protein for secretion (CAPS) family proteins (CAPS1 and CAPS2) are thought to act at the priming step of vesicle exocytosis by interacting with the SNARE proteins (syntaxin1/SANP25/VAMP2) and PIP_2 (James and Martin 2013). CAPS2 is the first molecule that has been shown to act as a positive regulator for BDNF release from cortical, hippocampal, and cerebellar neurons (Sadakata et al. 2007a; Sadakata et al. 2004). Notably, the deficiency of CAPS2 in mice showed impaired late phase of LTP and reduced number of cortical and hippocampal neurons, which was ameliorated by application of exogenous BDNF (Sadakata et al. 2007a; Sadakata et al. 2007b). CAPS2 promotes the depolarization-induced release of BDNF-pHluorin from main axons and their extra-synaptic sites of cultured mouse hippocampal neurons, in terms of kinetics, frequency, and amplitude (Sadakata et al. 2012; Shinoda et al. 2011) and axons of cerebellar granule cells (Sadakata et al. 2014; Sadakata et al. 2012). Another paralog CAPS1 is also involved in regulating the BDNF release. The knockdown of CAPS1 by shRNA eliminated the VAMP-2-dependent docking and evoked exocytosis of fusion-competent BDNF-EGFP vesicles in PC12 cells (Kabachinski et al. 2016). Acute single-cell knockdown of CAPS1 in cultured rat hippocampal neurons leads to a strong reduction in the number of fusion-competent secretory granules and to a significant decrease of released BDNF-GFP (Eckenstaler et al. 2016).

Conclusions

Even a trace amount of BDNF exerts a variety of crucial roles in the development and function of the nervous system. Therefore, the high-sensitive detection of in vivo local BDNF release used to be difficult in many cell types and neural circuits. Recent studies have overcome this difficulty by developing various sophisticated detection methods and

have documented kinetics of activity-dependent, Ca^{2+}-dependent release of BDNF from dendrites and axons in neurons and glial cells. Molecular mechanisms underlying dendritic-postsynaptic release and axonal-presynaptic release, however, remain to be elucidated. Several molecules such as CAPS2 and Syt-IV, which facilitates and inhibits BDNF release respectively, have been identified so far. Unraveling the release ratios of BDNF-associated peptides (BDNF, its precursor proBDNF and its pro-domain called BDNF pro-peptide) will also be informative to the understanding of their functional significances in brain function and dysfunction.

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Compliance with ethical standards

Ethics statement None.

Conflict of interest The authors declare that they have no conflict of interest.

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