ROLE OF NEUROTROPHINS IN CENTRAL SYNAPSE FORMATION AND STABILIZATION

Carlos Vicario-Abejón, David Owens‡, Ronald McKay‡ and Menahem Segal§*

The neurotrophins are best known for their ability to support neuronal survival and differentiation, but a role in synapse formation and plasticity has recently emerged. For central neurons, brain-derived neurotrophic factor can increase the number of excitatory and inhibitory synapses by regulating axonal morphology or by directly promoting synapse formation. In addition, neurotrophins promote the maturation and stabilization of the cellular and molecular components that are responsible for neurotransmitter release, and this ultimately leads to an increase in the number of functional synapses. These long-term structural and molecular changes are likely to be crucial not only during development, but also during synaptic plasticity in the adult.

**Group of Growth Factors in Vertebrate Development, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, E-28006 Madrid, Spain.*
‡Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 36/Room 3DO2, 36 Convent Drive, MSC 4092, Bethesda, Maryland 20892-4092, USA.
§Department of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel.
Correspondence to C.V.-A.
e-mail: cvicario@cib.csic.es
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Since the pioneering discovery of nerve growth factor (NGF), it has been thought that diffusible proteins, and in particular the neurotrophins, might have a role in regulating the initial steps in neuron–target interactions. In the peripheral nervous system (PNS), NGF released by cellular targets is crucial for the establishment and maintenance of synapses[1–3], and signalling mediated by the TrkB neurotrophin receptor is necessary for the maintenance of postsynaptic acetylcholine receptor clusters at the neuromuscular junction[4]. In the central nervous system (CNS), the neurotrophins NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) were initially characterized for their ability to support neuronal survival and differentiation[2]. However, more recent evidence supports a further role for neurotrophin signalling during central synapse formation, maturation and stabilization.

In this review, we discuss these new results, emphasizing the electrophysiological, cellular and molecular mechanisms that underlie the actions of neurotrophins. These are relatively 'slow' and long-lasting actions, and they are observed from several hours to several days after the addition of the neurotrophic factors. In this respect, they can be distinguished from the 'rapid' or acute (short-term) actions of BDNF on neuronal excitation and synaptic plasticity, which occur within minutes or even a fraction of a second after BDNF application[5–7]. Although rapid and slow neurotrophin-mediated modifications of synaptic function might share some underlying mechanisms[8], there are also differences. For example, whereas the chronic application of BDNF promotes the formation and maturation of GABA (γ-aminobutyric acid)-releasing inhibitory synapses between hippocampal or cerebellar neurons, the same neurotrophin depresses GABA-mediated synaptic transmission when it is acutely applied[9–12]. We refer the reader to previous articles that have addressed acute actions of neurotrophins that modulate synaptic transmission and plasticity[8,13–16].

In this review of the slower or long-term effects of neurotrophins, we discuss several issues. First, do neurotrophins change the number of synapses, or do they promote synapse maturation without increasing synaptic number? Second, do the neurotrophins act by regulating the morphology of dendritic and axonal arborizations, or do they act directly on synapses? Third, what are the structural, molecular and functional changes that are elicited by neurotrophins at synaptic terminals? Last, we ask whether ambient neuronal activity is a cofactor that is required for neurotrophins to mediate their actions during synapse formation, maturation and stabilization.

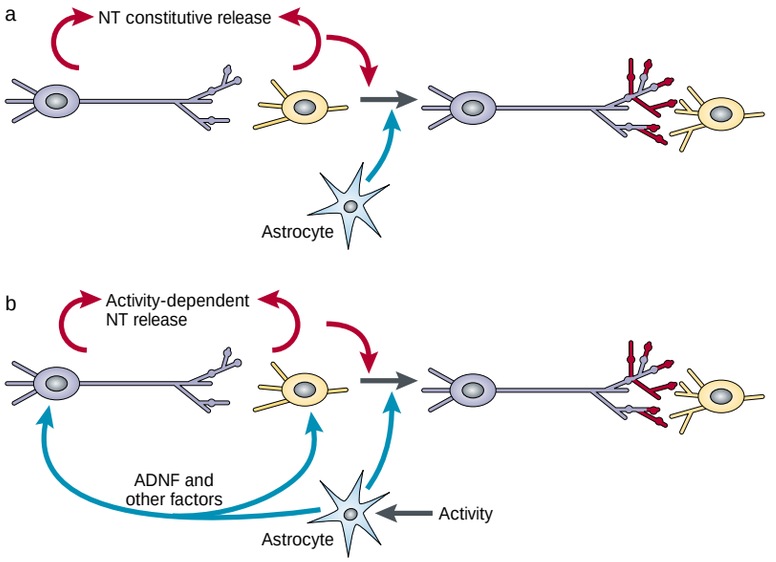

Figure 1 | **Neurotrophin effects during the establishment of synaptic circuits.** **a**,**b** | Neurotrophins (NT) can be released in an activity-independent manner (**a**) or in response to activity (**b**), by neurons and astrocytes, before or during target-contact initiation. Neurotrophins could increase the number of synapses by promoting axonal branching (red) and by directly inducing synapse formation (red boutons). Note that the increase in synapse number exceeds that of axonal branches. Neurotrophin release from neurons might be regulated by astrocyte-derived factors such as activity-dependent neurotrophic factor (ADNF) (**b**). This figure is based on several studies that are discussed in the main text.

Neurotrophins and their receptors

The neurotrophins bind to two types of transmembrane receptor protein to elicit biological responses: the tyrosine kinase Trk (tropomyosin receptor kinase) receptors and the p75 receptor. NGF is the preferred ligand for TrkA, whereas BDNF and NT4 bind preferentially to TrkB, and NT3 to TrkC, although this neurotrophin can also activate TrkA and TrkC in some cellular contexts[2]. They can all bind to the low affinity p75, but it has not been shown clearly whether this receptor is involved in central synapse development[17].

Initial studies of the expression of neurotrophins, and of TrkB and TrkC receptors in the CNS, indicated that these growth factors could act on postmitotic neurons to promote their survival and differentiation[2,18–21]. In addition, the observation that the TrkB and TrkC receptors were expressed in both axonal and dendritic compartments in hippocampal, cortical and cerebellar neurons implied that the neurotrophins could regulate synapse development and function[20,22–25]. This role was supported by the finding that neurotrophin-containing secretory granules are localized to synaptic sites[26,27].

NGF is synthesized and released by target cells in the PNS, and on binding to the TrkA receptor, it can be transported retrogradely along the axon to the cell body[28,29]. The situation might be more complex at CNS synapses, where retrograde, anterograde and trans-synaptic transport of neurotrophins have been described in both developing and mature neurons[30–36]. Neurotrophin synthesis, secretion and transport across synapses are tightly regulated by high-frequency activity during synaptic plasticity[33,34,37]. In addition, synaptic activity can regulate TrkB incorporation into the plasma membrane[38,39], enhancing the chances of ligand–receptor interactions, and consequently the rapid transmission of the signal[8,14].

Neurotrophins can also be released by neurons before or during the initiation of target contact, as well as by glial cells. In these situations, both constitutive secretion and regulated neurotrophin release can occur[40–44], promoting neuronal differentiation and synaptogenesis (FIG. 1). For example, hippocampal pyramidal neurons might use an activity-dependent NT3 AUTOCRINE loop to regulate their own differentiation[42]. PARACRINE modes of neurotrophin action might also influence synapse formation. A proportion of CNS synapses are contacted by astrocyte processes[45], and astrocytes potentiate synaptic efficacy and functional synapse formation[46–50]. Moreover, astrocytes secrete neurotrophins[44], indicating that the neurotrophins belong to a family of secreted factors that mediate crosstalk between neurons and glia during synaptogenesis. This phenomenon has been observed in hippocampal cell cultures, in which activity-dependent neurotrophic factor (ADNF), a protein that is secreted by vasoactive intestinal polypeptide (VIP)-stimulated astrocytes, acts postsynaptically to stimulate NT3 release. NT3 then acts presynaptically to promote the formation of functional glutamatergic synapses[47] (FIG. 1b).

So, the available data indicate that activity-independent and activity-dependent interactions between neurons and glia have a role in establishing the synaptic organization of the CNS. These interactions might be mediated, in part, through the regulation of neurotrophin release (FIG. 1). However, the exact role of neurotrophins in neuron–glia interactions remains to be established. In addition, proneurotrophins can be secreted and cleaved in the extracellular space[51], although the significance of this process for synapse formation and maturation is not known.

Synapse formation and stabilization

In principle, neurotrophins could act at two levels to regulate synapse number: by promoting synaptogenesis and/or by stabilizing existing synapses. In addition, they could influence the maturation of developing synapses. To assign an action to a particular neurotrophin during synapse development, it is necessary to have some understanding of synaptogenesis. Several stages can be distinguished during synapse formation: an initial stage, when the synaptic contact is first established but little pre- and postsynaptic differentiation has occurred ('nascent synapse'); a second stage, when the pre- and postsynaptic terminals can be clearly recognized at a morphological level; and maturation of the synapse, which involves the reorganization of pre- and postsynaptic components[52–55]. It is important to note that the presence of a morphological or structural synapse does not necessarily imply that the synapse is functional; that is, that synaptic transmission is actually taking place[55–59]. For this reason, we distinguish between the formation of morphological synapses, as assessed by electron microscopy (EM) or immunostaining of synapse-specific proteins,

AUTOCRINE
Describes an agent that acts on the cell that produced it.

PARACRINE
Describes a mechanism of signalling between cells that relies on the diffusion of signalling molecules through the intercellular spaces.

and the formation of functional synapses, as measured electrophysiologically.

Excitatory synapses. BDNF and NT3 exert specific and partially opposing effects on the dendritic and axonal morphology of central neurons, with the effects depending on the specific brain region, neuron type and cellular layer in which the neurons are located[56,60–63]. In addition, the TrkB ligands BDNF and NT4 regulate thalamic axonal segregation and OCULAR DOMINANCE COLUMN formation in the visual cortex[64–66]. These studies indicate that the regulation of dendritic and axonal morphology might be a mechanism through which the neurotrophins regulate the formation and stabilization of synaptic circuits in the CNS (FIG. 1). However, they do not show that the neurotrophins directly increase the number of synapses.

The first results to indicate that neurotrophins are involved in regulating synapse number came from studies of the brains of *Trkb*- and *Trkc*-knockout mice[21]. EM analysis revealed a moderate but significant reduction in the number of synapses in the postnatal hippocampus, as well as reduced axonal arborization. As the reduced synapse numbers were observed at a developmental age when synaptogenesis is normally taking place — postnatal days 12–13 (P12–P13) — these results are consistent with a role for neurotrophins in synapse formation, although a role in synapse stabilization cannot be ruled out at this stage.

In a recent *in vivo* study in the *Xenopus* visual system[67], BDNF was shown to augment the number of synapses, as evaluated by measuring the number of GREEN FLUORESCENT PROTEIN (GFP)-tagged clusters of synaptobrevin 2. Importantly, the GFP–synaptobrevin puncta showed a high degree of colocalization with the presynaptic markers FM4-64 and SNAP25 (synaptosome-associated protein, 25 kDa), confirming that they were localized at the synapse. The ultrastructural characteristics of the synapses were not analysed in this study. BDNF also enhanced optic axon arborization, but the increase in synapse number exceeded the increase in the number of axonal branches, indicating a further role for BDNF in synapse formation[67] (FIG. 1). In organotypic hippocampal slice cultures from P7 rats, BDNF augmented the dendritic spine density and excitatory synapse number in the CA1 region[24].

On the basis of these results, we suggest that neurotrophins can increase the density of excitatory synapses by two mechanisms. One mechanism depends primarily on its morphological effects on dendrites and axons (a permissive mechanism), and it probably works by enhancing the surface area of these elements, thereby increasing the number of potential contact sites. In the second mechanism, the neurotrophins promote synapse formation directly (an instructive mechanism) (FIG. 1). In addition, the neurotrophins might stabilize existing synapses[21,58] (see below).

So, increasing the level of BDNF expression has been shown to augment synapse formation, but what happens when the BDNF levels are reduced? Quantitative analysis of hippocampal synapses has not been reported in *Bdnf*-knockout mice, nor in a mouse with reduced TrkB levels in the forebrain[68–70]. However, a small reduction in the number of excitatory synapses from PARALLEL FIBRES to PURKINJE CELLS has been described in the cerebellum of mice that lack BDNF[25].

Recent results indicate that neurotrophins are key mediators of structural and functional synapse maturation; in particular, the conversion of presynaptically silent synapses to a functional state in culture[56,58]. Hippocampal cultures prepared from embryonic day 18 (E18) rat embryos develop 'normal' synaptic connections and express spontaneous network activity[56]. Cultured E16 neurons, which are largely glutamatergic, develop complex dendritic and axonal morphologies, contain EM-identified synapses, and express synaptic-vesicle-related proteins and glutamate receptors[47,56,58]. However, most of these synapses are functionally silent (FIG. 2a). Depolarization evokes action potentials, and the direct application of glutamate depolarizes the cells, but no functional synaptic connections are found. This phenomenon might be more widespread than was previously thought, as the presence of presynaptically silent synapses has also been observed in other neuronal systems[57,59,71,72]. In E16 hippocampal cultures, functional connectivity between the neurons, measured electrophysiologically or by uptake of the membrane-bound dye FM1-43, could be established by as little as a one-day exposure to BDNF or NT3 (FIG. 2a). By contrast, E18-derived neurons established functional synapses without the addition of exogenous BDNF or NT3, indicating that synaptic responses to neurotrophins are developmentally regulated and/or that endogenous neurotrophins are available to promote synapse formation in cells derived from this developmental stage.

Neurotrophins also seem to be necessary for the maintenance of functional synapses, because their removal produced a reduction in synaptic connectivity between cultured neurons[58]. Neurotrophin actions that promote the development of excitatory synapses have also been reported in other cultures of dissociated hippocampal cells and slices, reinforcing the idea that these factors regulate synapse maturation at the morphological, molecular and functional levels[24,73–76] (see below).

Inhibitory synapses. The neurotrophic effects of BDNF on GABA-expressing neurons have been well documented, both *in vivo* and *in vitro*[56,77–80]. In addition to these effects, several recent studies have shown that BDNF, but not NT3, promotes the formation of inhibitory synapses in hippocampal and cerebellar neurons in culture[56,81–84]. Furthermore, in the visual cortex and the cerebellum of transgenic mice that overexpress BDNF, the maturation of GABA innervation and the acquisition of inhibitory activity are accelerated[80,85].

The lack of an effect of NT3 in promoting the development of GABA synapses is not due to the absence of the TrkC receptor in inhibitory neurons[56] (FIG. 3a–d). Interestingly, NT3 increases dendritic length and branching in hippocampal GABA-expressing neurons in culture, but unlike BDNF, it does not elicit axonal changes[56] (FIG. 3e–g). These results indicate that the intracellular

OCULAR DOMINANCE COLUMN
One of a series of interdigitating bundles of axonal fibres in the visual cortex, containing afferents that represent one or other of the eyes.

GREEN FLUORESCENT PROTEIN (GFP)
A fluorescent protein that was originally isolated from the jellyfish *Aequorea victoria*. It can be genetically conjugated with proteins to mark them. The most widely used mutant, enhanced GFP, is excited at 488 nm and has an emission maximum at 510 nm.

PARALLEL FIBRES
Branches of the ascending axons of cerebellar granule cells. In the molecular layer of the cerebellar cortex, they run perpendicular to the planar Purkinje cell dendrites, with which they form so-called *en passant* synapses.

PURKINJE CELLS
Inhibitory neurons in the cerebellum that uses GABA as its neurotransmitter. Their cell bodies are situated beneath the molecular layer, and their dendrites branch extensively in this layer. Their axons project into the underlying white matter, and they provide the only output from the cerebellar cortex.

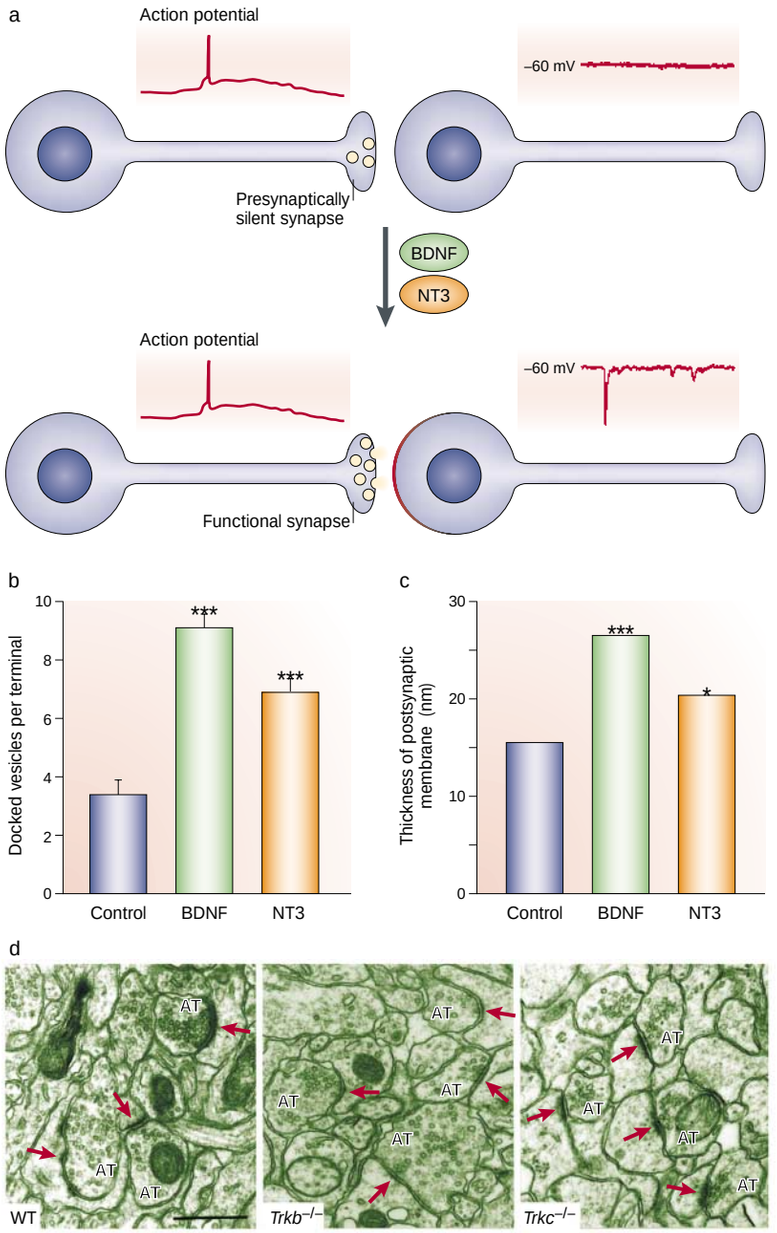

Figure 2 | **BDNF and NT3 functional and structural actions at excitatory synapses.** **a** | In the absence of neurotrophins, embryonic day 16 (E16) hippocampal neurons can develop to generate action potentials, postsynaptic responses to glutamate and synaptic structures, although most of the synapses are functionally silent (upper panel). Brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT3) increase the probability of neurotransmitter release at the presynaptic terminal and promote the establishment of functional synapses (lower panel). The presence of presynaptically silent synapses has also been observed in other neuronal systems[57,59,71,72]. Electrophysiological traces reproduced, with permission, from REF. 56 © 1998 Society for Neuroscience. **b** | In hippocampal cell cultures from E16 embryos, BDNF and NT3 produced three- and twofold increases in the number of docked vesicles, respectively. Reproduced, with permission, from REF. 58 © 2001 Federation of European Neuroscience Societies. An increase in the number of docked vesicles has also been described in hippocampal slice cultures treated with BDNF[24]. **c** | Neurotrophins also produced significant increases in the thickness of the postsynaptic membrane of cultured E16 hippocampal neurons. Reproduced, with permission, from REF. 58 © 2001 Federation of European Neuroscience Societies. **d** | Electron micrographs showing decreased number of synaptic vesicles and reduced thickness of postsynaptic membranes in hippocampal synapses from *Trkb*$^{-/-}$ and *Trkc*$^{-/-}$ mice. AT, axon terminal; WT, wild type. Reproduced, with permission, from REF. 21 © 1998 Society for Neuroscience.

signalling mechanisms that are activated by the TrkB and TrkC receptors might converge to promote dendritic growth, but that they are also divergent, in that BDNF also promotes axonal growth, and inhibitory synapse formation and maturation (FIG. 3g,h).

BDNF augments axonal length and the number of branches of GABA terminals in hippocampal cell and slice cultures, as well as in the visual cortex *in vivo*[56,74,80,81,84]. This might be a mechanism by which BDNF increases the number of GABA synapses (FIGS 1 and 3g). Moreover, analysis of cerebellar synapses has shown that TrkB receptor ligands can directly promote synapse formation, in addition to their effects on axonal branching[82,86]. Treatment of organotypic cerebellar cultures with blocking antibodies to BDNF and NT4 resulted in reduced inhibitory synapse formation, indicating that endogenous neurotrophins are required for inhibitory synapse development[82]. Accordingly, EM analysis of a *Trkb*-null mouse cerebellum showed a decrease in the number of GABA synapses, both during development and in the adult. These results indicate that TrkB receptor ligands regulate the formation of inhibitory synapses, although a role in synapse maintenance cannot be ruled out[86] (FIG. 3).

Several conclusions can be drawn from these studies. First, of the neurotrophins, only the TrkB ligands BDNF and NT4 promote the formation and stabilization of inhibitory synapses. Second, BDNF can increase the number of inhibitory synapses, either by regulating axonal morphology or by directly inducing synapse formation, and it can also stabilize existing synapses. Last, BDNF promotes the maturation of inhibitory synapses.

In addition to its effects on glutamate and GABA synapses, analysis of BDNF-deficient mice indicates that this neurotrophin is important for the maintenance of serotonergic (5-hydroxytryptamine or 5-HT) innervation and function in the adult brain[87].

Structural effects at synapses

EM has been used to evaluate the effects of the neurotrophins on the structure of the pre- and postsynaptic terminals of hippocampal and cerebellar neurons. During synaptogenesis, the total number of vesicles, as well as the number of DOCKED VESICLES, is a parameter that correlates with synapse maturation[52,54,88]. In hippocampal cultures from E16 embryos, BDNF and NT3 produced significant increases (three- and two fold, respectively) in the number of docked synaptic vesicles[58] (FIG. 2b). In hippocampal slice cultures from P7 neonates, BDNF augmented the number of docked vesicles with no change in the RESERVE POOL[24]. In accordance with the effects of exogenous neurotrophins, mice that lack BDNF, TrkB or TrkC receptors have a deficit in the number of docked synaptic vesicles in the hippocampus[21,68] (FIG. 2d). These studies all support a role for BDNF and NT3 in the maintenance of the docked vesicular pool at hippocampal excitatory synapses (FIG. 2a,b). A putative role for BDNF in synaptic vesicle biogenesis is also indicated by results that were obtained in hippocampal neuronal cultures, in which BDNF increased the total number of synaptic vesicles[58].

DOCKED VESICLES
The pool of synaptic vesicles that is available for rapid fusion with the presynaptic membrane in response to the arrival of a nerve impulse. These vesicles are docked to the membrane and a proportion of them are biochemically primed for release.

RESERVE POOL
A population of synaptic vesicles distal to the active zone that is recruited during periods of high-frequency stimulation.

ACTIVE ZONE
A portion of the presynaptic membrane that faces the postsynaptic density across the synaptic cleft. It constitutes the site of synaptic vesicle clustering, docking and transmitter release.

MINIATURE EXCITATORY POSTSYNAPTIC CURRENTS
Excitatory synaptic currents observed in the absence of presynaptic action potentials. They are thought to correspond to the discharge of single vesicles.

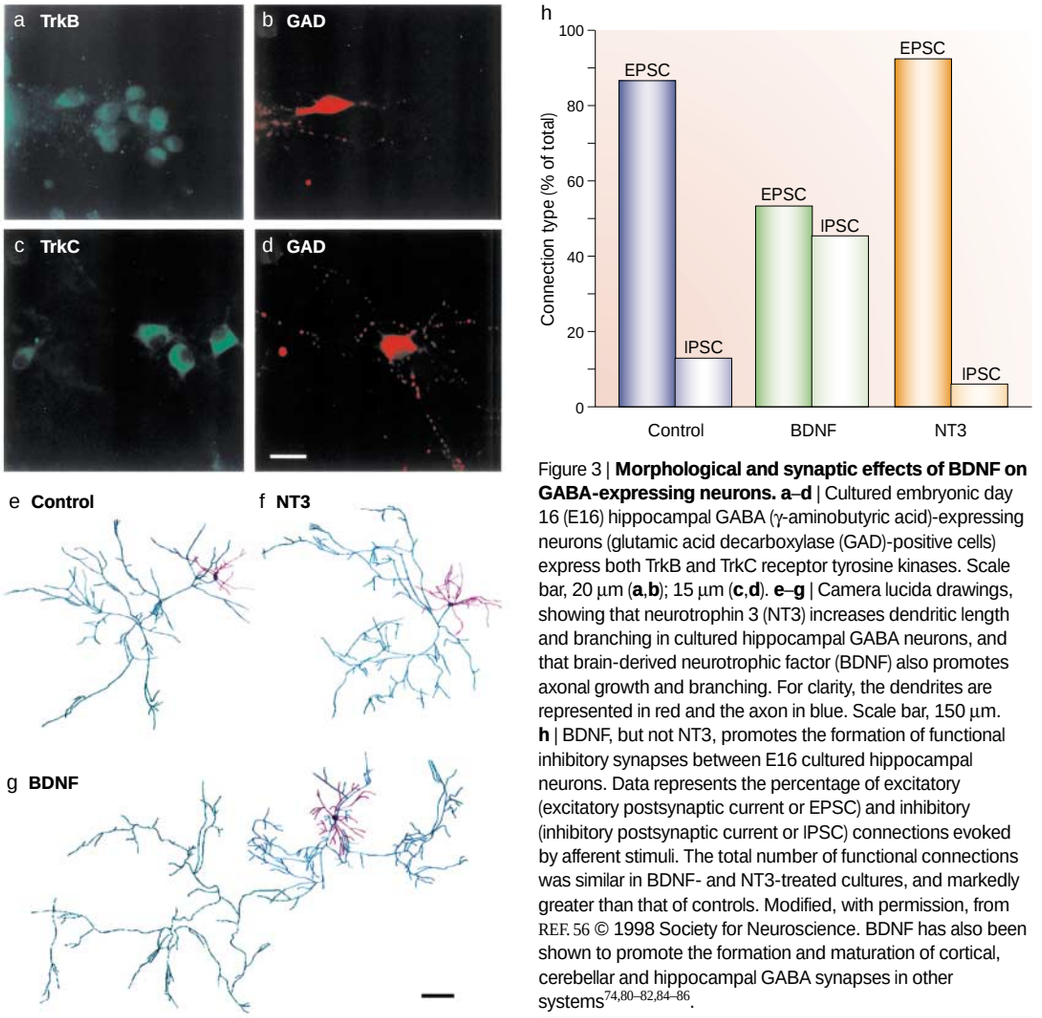

Figure 3 | **Morphological and synaptic effects of BDNF on GABA-expressing neurons. a–d** | Cultured embryonic day 16 (E16) hippocampal GABA (γ-aminobutyric acid)-expressing neurons (glutamic acid decarboxylase (GAD)-positive cells) express both TrkB and TrkC receptor tyrosine kinases. Scale bar, 20 μm (**a**,**b**); 15 μm (**c**,**d**). **e–g** | Camera lucida drawings, showing that neurotrophin 3 (NT3) increases dendritic length and branching in cultured hippocampal GABA neurons, and that brain-derived neurotrophic factor (BDNF) also promotes axonal growth and branching. For clarity, the dendrites are represented in red and the axon in blue. Scale bar, 150 μm. **h** | BDNF, but not NT3, promotes the formation of functional inhibitory synapses between E16 cultured hippocampal neurons. Data represents the percentage of excitatory (excitatory postsynaptic current or EPSC) and inhibitory (inhibitory postsynaptic current or IPSC) connections evoked by afferent stimuli. The total number of functional connections was similar in BDNF- and NT3-treated cultures, and markedly greater than that of controls. Modified, with permission, from REF. 56 © 1998 Society for Neuroscience. BDNF has also been shown to promote the formation and maturation of cortical, cerebellar and hippocampal GABA synapses in other systems[74,80–82,84–86].

In the cerebellum, by contrast, the targeted deletion of BDNF reduces the proportion of docked synaptic vesicles at excitatory synapses between parallel fibres and Purkinje cells by augmenting the number of synaptic vesicles distant from the ACTIVE ZONE, rather than by decreasing the absolute number of docked vesicles[25]. In spite of differences in the ultrastructural features observed in the hippocampus and the cerebellum of BDNF mutant mice, in both cases the net effect is impaired function of the presynaptic terminal[25,68]. This indicates that both absolute and relative numbers of docked synaptic vesicles are important parameters in regulating presynaptic function. Together, the data obtained from hippocampal and cerebellar synapses indicate that endogenous BDNF regulates the mobilization of vesicles from a reserve pool to a docked synaptic pool (FIG. 4). They also imply that neurotrophins regulate synaptic vesicle dynamics through slightly different mechanisms in the two brain regions.

Functional effects of neurotrophins

Excitatory synapses. The structural effects of neurotrophins at presynaptic terminals, as described above, have functional correlates. Several assays have been used to evaluate pre- and postsynaptic functional modifications. In E16 hippocampal neurons in culture, and in P7 hippocampal slices, neurotrophins were shown to greatly increase the frequency of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-receptor-mediated MINIATURE EXCITATORY POSTSYNAPTIC CURRENTS (mEPSCs) evoked by hyperosmotic stimulation. However, the effects on mEPSC amplitudes were minimal[24,56,58]. A change in the frequency of miniature postsynaptic currents (mPSCs) is usually attributed to a change in the probability of neurotransmitter release, whereas a change in the amplitude of the mPSC could be caused by a change in the concentration of transmitter within a vesicle or by a change at the postsynaptic site. This finding is therefore consistent with the idea that the neurotrophins induce presynaptic modifications. In addition, α-latrotoxin, a potent neurotoxin that triggers neurotransmitter release from presynaptic nerve terminals[89], was virtually ineffective in control cells, but it stimulated synaptic events in neurotrophin-treated hippocampal cells[58]. In agreement with results from both assays, FM1-43 was taken up actively by presynaptic terminals in neurotrophin-treated hippocampal cultures,

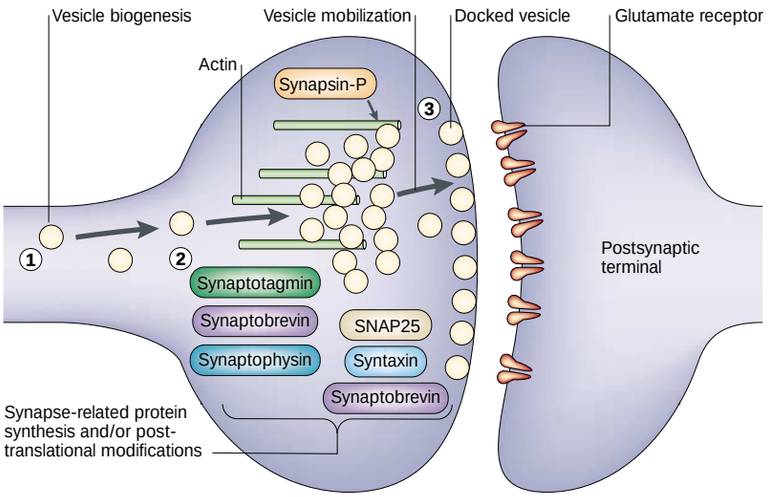

Figure 4 | **Neurotrophin-mediated maturation of neurotransmitter-release machinery at excitatory presynaptic terminals.** During maturation of the presynaptic terminal, neurotrophins might regulate synapse-related protein synthesis and/or modulate protein–protein interactions by regulating post-translational modifications. These actions could lead to an increase in protein incorporation into synaptic vesicles during vesicle biogenesis (**1**), vesicle accumulation into the reserve pool (**2**) and/or stabilization of the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) complex — SNAP25 (synaptosome-associated protein, 25 kDa), syntaxin and synaptobrevin. Brain-derived neurotrophic factor (BDNF) might also activate synaptic vesicle mobilization from a reserve pool to a docked pool, possibly through the BDNF-mediated regulation of synapsin 1 phosphorylation (**3**). This model is based on several studies that are discussed in the main text.

but was taken up inefficiently in controls[58,75]. All the above results are consistent with a presynaptic locus of neurotrophin action to increase the pool of docked synaptic vesicles, which is essential for rapid synaptic transmission (FIG. 2a,b). A correlation between the size of the pool of docked vesicles and the probability of mEPSC events has been shown[90,91].

Consistent with the results obtained with exogenous neurotrophins, functional properties such as high-frequency stimulation and PAIRED-PULSE FACILITATION, which depend largely on presynaptic mechanisms, are impaired in hippocampal and cerebellar synapses of *Bdnf*-knockout mice[25,68]. Moreover, a decrease in the mEPSC frequency in hippocampal cells that were treated with TrkB receptor bodies (TrkB–IgG) has been reported[74], indicating that endogenous BDNF can modulate presynaptic mechanisms. Further evidence of a presynaptic locus of neurotrophin action during synapse formation came from experiments in which postsynaptic NMDA (*N*-methyl-D-aspartate) and non-NMDA glutamate receptors were blocked with AP5 (D(–)-2-amino-5-phosphonovaleric acid) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), respectively. It was found that this treatment did not prevent the formation of functional synapses that is induced by neurotrophins in E16 hippocampal neurons[58].

However, other data indicate that the actions of the neurotrophins during synapse development are not mediated exclusively by the presynaptic terminal. For example, chronic treatment of hippocampal or cortical cultures with BDNF regulates excitatory synaptic transmission by increasing[73,74,92] or decreasing[92] the amplitude of AMPA-mediated mEPSCs, consistent with a postsynaptic mechanism. Although the correlation between structural and functional synapses was not analysed in these studies, other experiments showed that neurotrophins regulate the maturation of the POSTSYNAPTIC DENSITY of hippocampal synapses[21,58] (FIG. 2c,d), and the expression of AMPA receptor subunit 2 (GluR2) in cortical neurons, both *in vivo* and in culture[93,94].

The effects of BDNF on AMPA-mediated mEPSC amplitudes in visual cortical pyramidal neurons seem to be regulated by the postsynaptic neuron[92,95]. This has been observed in cultures, in which chronic treatment with BDNF decreased the mEPSC amplitudes recorded from other pyramidal neurons, and increased the mEPSC amplitudes recorded from interneurons. These results indicate that other factors released from the target neurons might modulate the actions of BDNF during circuit formation[96]. They also suggest that BDNF might differentially regulate AMPA receptor expression on pyramidal cells and interneurons.

So, although a presynaptic locus of action seems to be crucial for the promotion of excitatory synaptic development by the neurotrophins, a postsynaptic mechanism is also plausible. The two possibilities are not mutually exclusive, as maturation of the neurotransmitter-release apparatus might in turn promote further maturation of the postsynaptic site[59].

Inhibitory synapses. The mechanisms by which BDNF promotes the maturation and stabilization of inhibitory synapses have been studied in less detail than those of excitatory synapses. The observation that BDNF increases the frequency of miniature inhibitory postsynaptic currents (mIPSC) in cultured hippocampal neurons supports a presynaptic mechanism of action[56,74]. Consistent with this, BDNF enhances potassium-evoked GABA release from cultured hippocampal neurons, indicating that BDNF might positively influence the GABA-release machinery[84]. In addition, BDNF might increase GABA synthesis by enhancing the expression of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis, as well as GABA uptake at GABA terminals[74,80,81,84,86,97]. An increase in the GABA content of synaptic vesicles could in turn enhance mIPSC amplitudes[56,85]. Although these are probably the predominant mechanisms of BDNF action during the maturation of GABA-releasing synapses, direct postsynaptic mechanisms might also be involved, such as a BDNF-induced upregulation of the $GABA_A$ receptor subunits β2 and β3, as seen in hippocampal neurons[84].

Molecular mechanisms

A clear correlation between morphological, functional and molecular mechanisms of neurotrophin action at synapses has yet to be established. However, recent data have provided some important clues about the underlying molecular mechanisms of neurotrophin-induced synapse maturation.

PAIRED-PULSE FACILITATION
When two stimuli are delivered to an axon in quick succession, the response elicited by the second stimulus is larger than that evoked by the first. This phenomenon is termed paired-pulse facilitation, and it is believed to depend on residual calcium that enters the presynaptic terminal.

POSTSYNAPTIC DENSITY
An electron-dense thickening underneath the postsynaptic membrane that contains receptors, structural proteins linked to the actin cytoskeleton and signalling elements, such as kinases and phosphatases.

N-ETHYLMALEIMIDE-SENSITIVE FUSION PROTEIN
An ATPase that is a key component of the membrane fusion machinery.

SNARE PROTEINS
A family of membrane-tethered coiled-coil proteins that regulate exocytotic reactions and target specificity in vesicular fusion processes. SNARE stands for 'soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor'.

Influence on the neurotransmitter-release machinery. In neurons, α-latrotoxin stimulates neurotransmitter release by synaptic vesicle exocytosis in a calcium-independent manner. This action involves direct stimulation of the neurotransmitter secretory apparatus at the synapse. The soluble *N*-ETHYLMALEIMIDE-SENSITIVE FUSION PROTEIN (NSF)-attachment protein receptor (SNARE) PROTEINS — synaptobrevin, syntaxin and SNAP25 — are required for α-latrotoxin action[89] (FIG. 4). The fact that neurotrophins can induce α-latrotoxin responses in E16 hippocampal neurons indicates that these growth factors regulate the proteins that control neurotransmitter release[58]. The maturation of the neurotransmitter-release machinery has a prominent role in the transformation of immature synaptic contacts into mature functional synapses[57,59]. Moreover, the SNARE-dependent mechanism of neurotransmitter release seems to be crucial for AMPA-type glutamate receptor activation during the functional maturation of synapses in hippocampal cultures[59].

SNARE protein expression has been analysed in neurotrophin-treated cultures, and in *Bdnf*-, *Trkb*- and *Trkc*-knockout mice. Syntaxin 1 levels were not modified by neurotrophin treatment in hippocampal cell cultures[56], nor were the levels of synaptobrevin or SNAP25 (C.V.-A. *et al.*, unpublished observations). Accordingly, syntaxin 1 and SNAP25 expression were unaltered in the hippocampus of *Bdnf*-knockout mice[68]. Immunocytochemical studies of hippocampal sections from *Trkb*-null mice revealed a moderate reduction in the expression of syntaxin and SNAP25, but not of synaptobrevin 2 (REF. 21). Other studies reported significant increases in synaptobrevin levels in BDNF-treated cortical neurons[98], and in hippocampal cells[84] and slices[76], although the increase in the latter study was not blocked by inhibitors of protein synthesis[76]. However, it should be stressed that, in some studies, the levels of synaptic proteins were measured in cultured cells before synapse formation[98], or when the presence of synapses had not been corroborated at the EM or electrophysiological levels[84]. Therefore, the relationship between the neurotrophin-induced protein increases and the actual process of synapse maturation was not strictly established[84,98].

So, functional analysis indicates that the neurotrophins regulate synaptic proteins that control neurotransmitter release[24,56,58], but biochemical results indicate that this regulation is not attributable to an overall change in the levels of these synapse-related proteins. Instead, the neurotrophins might modulate protein–protein interactions by regulating post-translational modifications that lead to stabilization of the SNARE complex, and might also control protein incorporation into synaptic vesicles[56,68,76] (FIG. 4). In keeping with this proposal, *Bdnf*-knockout mice showed decreases in synaptobrevin levels in synaptosomes, but not in whole hippocampal extracts, indicating that BDNF might regulate synaptobrevin incorporation into synaptic vesicle membranes[68] (FIG. 4).

In addition to synaptobrevin, the vesicular proteins synaptophysin, synaptotagmin and synaptic vesicle glycoprotein 2 (SV2) are inserted into the synaptic vesicle membrane (FIG. 4), and have roles in synaptic vesicle maturation and neurotransmission[99–104]. Confocal analysis of immunostained hippocampal cultures, and Western blot analysis of whole-cell protein extracts from neurotrophin-treated cells, showed no differences in the amount or distribution of synaptotagmin, synaptophysin and SV2 (REF. 56) in these cells compared with controls. Similar to the results obtained for synaptobrevin, *Bdnf*-knockout mice showed decreases in synaptophysin levels in synaptosomes, but not in whole hippocampal extracts, indicating that BDNF influences synaptophysin incorporation into synaptic vesicles[68] (FIG. 4). Interestingly, a protein-synthesis-dependent increase in synaptotagmin levels was observed after the treatment of hippocampal slices with BDNF[76]. So, although most data concur with a role for BDNF in regulating post-translational modifications of presynaptic proteins[56,68,76], the effect on synaptotagmin levels might be of translational origin. Interestingly, the BDNF effect requires gating by cyclic AMP, as the BDNF-induced synaptotagmin increase was blocked by inhibiting the cAMP-dependent protein kinase (PKA) pathway, although BDNF itself did not activate PKA[76,105].

Influence on the postsynaptic terminal. In addition to their roles in regulating neurotransmitter release, the neurotrophins might also regulate molecular events at the postsynaptic terminal. For example, BDNF has been shown to regulate the surface expression of AMPA receptor subunits at cortical neuronal membranes, probably through post-transcriptional and/or post-translational mechanisms[93,94]. BDNF induces rapid surface translocation of the GluR2 subunit, apparently by promoting an interaction between GluR2 and NSF. This process requires intracellular calcium influx and can be prevented by blocking the activity of protein kinase C[94].

NT3 seems to control the stability of the NMDA receptor subunits NR2A and NR2B in cultured hippocampal neurons, although the underlying mechanisms still need to be explored[47]. However, the total levels of GluR1 and NR1 do not seem to be under the control of neurotrophins[56,84].

Furthermore, BDNF might influence the expression of GABA receptors, as long-term BDNF treatment has been found to upregulate the $GABA_A$ receptor subunits β2 and β3 in hippocampal neurons[84].

Signal-transduction mechanisms. The increase in the number of docked vesicles at presynaptic terminals of hippocampal synapses that is produced by BDNF and NT3 (REFS 24,58) (FIG. 2a,b), in addition to the increase in the number of synaptic vesicles that are distant from the active zone in cerebellar synapses of *Bdnf*-knockout mice[25], indicate that neurotrophins activate synaptic vesicle mobilization from a reserve pool to a docked pool (FIG. 4). Synapsin 1 is an important synaptic-vesicle-associated protein, and its phosphorylation regulates synaptic vesicle mobilization and neurotransmitter release[106,107]. A deficit of synapsin 1 and synapsin 2 in double-knockout mice results in a reduction in the number of synaptic vesicles at hippocampal synapses, and also causes a delay in synaptogenesis[88,108–110].

Interestingly, synapsin 1 phosphorylation through the mitogen-activated protein kinase (MAPK) pathway is a mechanism by which BDNF (but not NT3 or NGF) acutely stimulates neurotransmitter release[111,112]. Synapsin 1 is necessary for the action of BDNF, because the stimulation of glutamate release by the neurotrophin is strongly attenuated in mice that lack synapsin 1 and/or synapsin 2 (REF. 112). It is therefore possible that changes in the phosphorylation state of synapsin 1 that are elicited by long-term BDNF treatment can regulate synaptic vesicle docking or mobilization. Indeed, the presence of a basal level of MAPK-dependent synapsin 1 phosphorylation has been shown in synaptosomes derived from the cerebral cortex, and this might be under tonic BDNF regulation[112]. In addition, active MAPK is necessary for the neurotrophin-induced increase in the number of P/Q-type calcium channels in hippocampal neurons, which is involved in neurotransmitter release, as these channels are associated with the release machinery at some central synapses[113–115]. So, it seems that MAPK might be a mediator of neurotrophin actions during central synapse maturation, as well as during axonal growth[116,117].

A further signalling pathway that is involved in axonal growth control, the phosphoinositide 3-kinase (PI3K) pathway[38,116,117], mediates the acute NT3 potentiation of neurotransmitter release in developing *Xenopus* spinal neurons in culture[118]. In addition, this signalling pathway might have a role in the control of basal transmitter release[118], possibly in part by promoting the reorganization of cytoskeletal proteins[117,119]. An interesting possibility is that BDNF and NT3 use both common and distinct signalling pathways to promote the maturation of the neurotransmitter-release apparatus at different synapses[112,118]. In support of this, it was recently reported that a docking site for the Shc ADAPTOR PROTEIN is necessary for TrkB- but not for TrkC-activated signalling pathways that are involved in the maintenance of target innervation by sensory neurons[120].

In addition to the MAPK and PI3K pathways, neurotrophins might use distinct pathways to specifically affect pre- and postsynaptic functions. It has been recently reported that the depolarizing effects of BDNF and NT4 on central neurons require the TrkB-mediated activation of the TETRODOTOXIN (TTX)-insensitive sodium channel $Na_v1.9$ (REF. 121). Interestingly, this channel might also be involved in the promotion of neurite outgrowth by BDNF in cultured hippocampal neurons[122].

The role of synaptic activity

Many studies support a role for synaptic activity in regulating the refinement and precise pattern of synaptic connections during development, and these actions are mediated, at least in part, by neurotrophins. There is also evidence that activity is required for the neurotrophin-mediated regulation of synaptic transmission and plasticity[8].

In the visual cortex, it has been reported that the dendritic-growth-promoting effects of neurotrophins require activity[123]. Presynaptic depolarization (and/or increased levels of cAMP) facilitate neurotrophin-induced synaptic potentiation at developing neuromuscular synapses[124]. However, BDNF can promote the arborization of optic nerve axons in the absence of activity[125], and NT4 can promote connectivity in the visual cortex independently of activity[126]. These results indicate that different modes of interaction exist between activity and neurotrophins during circuit formation and plasticity.

Studies in cultured hippocampal, cortical and cerebellar neurons have addressed the question of whether chronic neurotrophin treatment requires concurrent action potential activity to promote synapse formation and maturation[58,74,82,92]. The primary finding is that neurotrophins can promote the development of excitatory and inhibitory synapses in the presence of TTX, indicating that neurotrophins do not require action potential invasion of the presynaptic terminal to promote the maturation of these synapses. In addition, TTX decreased the expression of GABA in cortical cultures, although the numbers of GABA-expressing neurons were augmented by BDNF, even in the presence of the toxin[97]. Furthermore, the blockade of AMPA/kainate-type glutamate receptors, NMDA-type glutamate receptors and L-type calcium channels did not inhibit functional synapse formation by neurotrophins[58].

These data indicate that, during the initial events of synaptic circuit formation, neurotrophins can promote the development of excitatory and inhibitory synapses in the absence of activity (FIG. 1). This is consistent with *in vivo* studies, in which synapse formation has been described in situations where activity is blocked[127,128]. However, spontaneous activity might have an instructive role for the development of retinogeniculate connectivity[129], and for the promotion of retinal ganglion cell axonal growth by neurotrophic factors[130]. In the developing hippocampus, spontaneous activity seems to regulate NT3 release from pyramidal neurons before target innervation[42], and BDNF and activity might regulate the density of inhibitory synapses in culture[81]. So, a scenario can be proposed in which molecular cues, including the neurotrophins, control neuronal circuit formation and maturation by activity-independent mechanisms, as well as by interacting with activity-dependent processes (FIG. 1).

Concluding remarks

There is increasing evidence that TrkB (BDNF and NT4) and TrkC (NT3) ligands promote the formation, maturation and stabilization of glutamatergic and GABA synapses in the CNS. This raises the possibility that local neurotrophin levels might regulate the balance between excitatory and inhibitory transmission during the early stages of synaptic circuit formation. The neurotrophins seem to act predominantly at presynaptic sites, although postsynaptic effects are also involved.

Although considerable progress has been made in this field, the underlying cellular and molecular mechanisms that mediate the actions of neurotrophins at synapses remain unclear. There is also a lack of understanding of the signalling mechanisms that mediate the biological responses that are elicited by different neurotrophins in excitatory and inhibitory neurons. In the

ADAPTOR PROTEIN
A protein that contributes to cellular function by recruiting other proteins to a complex. Such molecules often contain several protein–protein interaction domains.

TETRODOTOXIN
A neurotoxin derived from the *Fugu*, or puffer fish, that specifically and reversibly blocks voltage-gated sodium channels.

future, it will be important to visualize *in vivo* the relationship between constitutive and activity-regulated secretion of neurotrophins, and the formation and stabilization of new and existing synapses. By elucidating the mechanisms that regulate the formation and maintenance of synapses by neurotrophins, we will gain a better understanding of the molecular nature of synaptic development and plasticity.

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DATABASES

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