DISCUSSION

This study was undertaken to investigate the role of BDNF and PRG-1 in dendritogenesis and synaptogenesis in hippocampal neurons. A defined cellular source of EGFP, BDNF::EGFP or PRG1::EGFP was created by transfecting hippocampal or entorhinal cortical neurons in culture. We report the following new results: 1) Neurons expressing BDNF displayed changes in dendrite morphology: they exhibited weaker dendrite elongation (fewer dendritic trees with a length >50 μm), but stronger dendrite initiation (a larger number of primary dendrites and higher-order branches). 2) Neurons expressing BDNF also attracted a larger total number of synaptic terminals. 3) A cellular source of BDNF produced a stronger upregulation of synaptic terminal numbers than ambient BDNF at high concentration (100 ng/ml). 4) Glutamatergic and GABAergic synaptic terminals reacted in a differential manner to the postsynaptic BDNF. The strength of the glutamatergic input increased, while the strength of GABAergic input decreased. 5) The upregulation of glutamatergic synaptic input by the postsynaptic BDNF required TrkB activity. 6) The transfected BDNF (t-BDNF)-induced downregulation of GABAergic synaptic terminal numbers and dendrite elongation were also prevented by block of TRkB. Moreover, these two suppressive actions of t-BDNF were precluded by a cocktail of GluR blockers suggesting that the suppression was associated with t-BDNF-stimulated GluR activity. 7) Application of a function-blocking antibody against p75 failed to prevent the changes in synapse numbers and dendrite length, but it reduced the branching response to BDNF transfection. 8) PRG-1-overexpression attenuated the LPA-mediated decrease in synapse number. Suppression of PRG-1 expression reduced the number of glutamatergic synaptic terminals.

Based on these results it is proposed (see cartoons in Figs. 25, 26) (i) that postsynaptic expression and release of BDNF and its binding to presynaptic TrkB receptors boosts the glutamatergic synaptic input. The contribution of p75 to this glutamatergic upregulation is small, if at all existing. (ii) The arrest of dendritic length growth and the downregulation of GABAergic synaptic input are consequences of the enhanced glutamate receptor activity rather

than a result of direct action of BDNF on TrkB receptors on the postsynaptic dendrites or presynaptic GABAergic terminals, respectively. (iii) Dendrite initiation is controlled by p75 and reflects the level of ambient neurotrophin concentration (and the action of other ligands of p75). In this process, the impact of glutamatergic synaptic input is less obvious. iv) PRG-1 can act as membrane-bound postsynaptic stabilizer of glutamatergic synaptic terminals.

1. EXPERIMENTAL MODEL

In the present study, transfection of BDNF::EGFP into single neurons in low density cultures system from *bdnf-/-* hippocampi offered an opportunity to explore the significance of extracellular gradients of BDNF on dendrite development and synaptogenesis via autocrine and retrograde mechanisms. The expression of BDNF::EGFP in only a small fraction (0.02%) of neurons isolated from each other by a distance of at least 2.2 mm, was assumed to set up a local source of BDNF. This experimental model has the advantage that one can distinguish between the changes induced by pre- or postsynaptic source of BDNF. Here we only describe the latter.

Previous studies which aimed at resolving the question that how BDNF acts to regulate dendrite geometry and synaptic transmission used other experimental approaches, such as addition of exogenous BDNF or scavenger antibodies, BDNF overexpression or BDNF gene knockout. Exogenous addition of BDNF, NT-3 and NT-4/5 exerted layer-specific effects on dendrite growth of pyramidal neurons (McAllister et al., 1997; Niblock et al., 2000). Neutralizing endogenous neurotrophins also exerted changes in dendrite development (McAllister et al., 1997). Additionally, BDNF overexpression through particle-mediated gene transfer in ferret visual cortical slices greatly altered the morphology of dendrites and spines (Horch et al., 1999). However, exogenous applications or overexpression of BDNF create dosages which are not only non-physiological but also at variance with the assumptions of the classical target-derived neurotrophic factor hypothesis where neurons are thought to compete for very small amounts of neurotrophins released by their synaptic targets (Levi-Montalcini and Hamburger, 1951; Bothwell, 1995). In accordance with this neurotrophic factor concept, Gallo and coworkers presented an *in vitro* system showing growth cone turning of the dorsal root ganglion (DRG) axons toward NGF-coated beads functioning as a localized source of NGF (Gallo et al., 1997, Gallo and Letourneau, 1998). Later, using compartmentalized

diffusion chambers for cell cultures, it was shown that extracellular concentration gradients are indeed important for neurite outgrowth in PC12 cells and DRG axons (Cao and Shoichet, 2001, 2003). However, exogenous NT sources as well as overexpression in wild type neurons may in fact produce a diffuse distribution of the NTs. Such methods of treatment could therefore account for the contradictory results described so far.

2. LOCAL EFFECTS OF TRANSFECTED BDNF VERSUS GENERAL EFFECTS OF EXOGENOUS BDNF

2.1. Changes in dendrite morphology: effects of transfected BDNF vs exogenous BDNF

Our experiments showed that creating a local source of BDNF by BDNF::EGFP transfection in single primary hippocampal neurons from *bdnf-/-* mice exerted a marked effect on dendrite morphogenesis. After 16 h of BDNF::EGFP expression, the hippocampal neurons projected additional primary dendrites of short length and showed enhanced dendritic branching. Moreover, BDNF::EGFP transfected neurons displayed an arrest of dendritic elongation. Added exogenous BDNF (100 ng/ml) induced similar changes but to a lesser degree. For instance, there was an increase in the number of dendritic branch points by +76.4 ± 5.0% in BDNF::EGFP-transfected neurons, as compared to +37 ± 4.9% in non-expressing neurons exposed to BDNF.

A change in dendrite development was already reported in previous studies based on BDNF-overexpression. The number of proximal dendrites increased at the expense of more distal dendritic branches (Horch et al., 1999). But, the experiments in the present study demonstrated an even more pronounced difference in the dendritic morphology, and it also became clear that the length of the dendrites decreased. Thus, dendrite branching and elongation are differentially controlled by BDNF. And, since the present comparison was between BDNF-transfected neurons and the neurons entirely lacking BDNF, it could be concluded that the effects obtained with local cellular BDNF and diffuse distribution of BDNF are similar in direction, but different in magnitude.

2.2. Changes in the number of synaptophysin I positive terminals: effects of transfected BDNF vs exogenous BDNF

Comparison of synapse numbers in the two populations of BDNF-supplemented neurons also revealed a more drastic change effected by BDNF::EGFP than the added BDNF. BDNF::EGFP-transfected neurons showed a +93.4 ± 4.8% increase in the number of Syp I-positive terminals but the neurons treated with added BDNF showed a +40.8 ± 6.7% increase. A more drastic difference was revealed when we compared these two groups to the wild type neurons. Addition of BDNF in *bdnf-/-* cultures could not raise the number of synaptic connections to the level in *bdnf*+/+ cultures (74.8 ± 3.6% of wildtypes), whereas transfection of BDNF::EGFP into a few neurons in the *bdnf-/-* cultures completely recovered the wild-type level of synapse numbers (102.8 ± 2.6% of wildtypes, Fig. 13). From this comparison, it was concluded that the magnitude of effects produced by BDNF application depends on its route of action and delivery.

A previous study of transgenic mice with BDNF overexpression in sympathetic neurons showed a two- to fourfold increase in synapse number as obtained from electron microscopic counts of synaptic inputs (Causing et al., 1997). Indirect evidence that neurotrophins promote synapse formation has, in fact, been provided earlier by recording mPSC activity at the neuromuscular junction of *Xenopus* (Lohof et al., 1993; Wang et al., 1995). Here, supporting evidence was provided by Dr. Henneberger, Charité, Berlin, who performed mPSC recordings from sister cultures available from the preparations used for morphometry. It was found that in BDNF-transfected neurons the fraction of mEPSCs in the total miniature activity was higher than in EGFP-expressing neurons lacking BDNF (Fig. 24).

3. CHANGES IN THE EXCITATORY-TO-INHIBITORY RATIO OF SYNAPTIC TERMINAL NUMBER

The experiments demonstrated an upregulation in the number of glutamatergic and a downregulation in the number of GABAergic synaptic terminals after transfection of BDNF::EGFP in individual *bdnf-/-* neurons. In general, BDNF could regulate the number of synapses contributing to the activity of the postsynaptic neuron in four mutually not exclusive ways: first, by attracting or repelling approaching afferents, second, by stabilizing or

destabilizing the existing synapses, third, by affecting the transmitter release, and, fourth, by changing the number, stability or activity of respective transmitter receptors.

3.1. Possible mechanisms of BDNF action on synaptic terminal numbers

Selective attraction. The BDNF::EGFP transfection experiments suggest that postsynaptic BDNF exerts its effects on synapse numbers by binding to NT receptors of either or both types of axon terminals, which does not exclude retrograde transport throughout the entire presynaptic neuron and/or an autocrine action on postsynaptic NT receptors. The standard dogma of retrograde signaling entails a postsynaptic production of a signal, either constitutive or regulated (for example, by activity), which in our case is the extracellular gradient produced by BDNF release from the transfected neuron. This can produce transfer of the soluble factors through the plasma membrane or interaction of membrane-bound components. An argument in favor of a role of BDNF as an attractive or a repulsive signal, depending on the type of synapse, is the presence of the respective presynaptic receptors, i.e. TrkB. Such receptors do exist in the mouse hippocampus (Du and Poo, 2004). TrkB activation could regulate axonal elaboration and complexity of hippocampal afferents but not the ingrowth (Martinez, 1998). Thus, BDNF signaling may exert both tropic and trophic influences on the approaching axon terminals.

Chemotropic guidance of axons through gradients of neurotrophins was already shown to play a role in synapse development (Gunderson and Barrett, 1979; Ming et al., 1997; Song et al., 1997). Several previous studies in the CNS have shown that exogenously applied neurotrophins may regulate axonal and collateral branching. *In vivo* infusion of BDNF, but not other neurotrophins, into the optic tectum of *Xenopus* tadpoles stimulated rapid increase in branching and complexity of retinal ganglion cell axons (Cohen-Cory and Fraser, 1995).

Suppression of contact-formation may also take place through a BDNF gradient. But a role for neurotrophins in this function has not yet been documented. The present study does not exclude the possibility that BDNF guides specific presynaptic terminals to form or to avoid forming synaptic connections. If this idea applies, GABAergic axonal terminals might sense the presence of BDNF as an inhibitory cue and therefore, turn away from the BDNF expressing postsynaptic neuron. And this response would be mediated by TrkB receptors as the block of the latter prevented the downregulation of GABAergic synaptic terminal

numbers. However, at present this study favors another idea to explain the t-BDNF-induced down-regulation of inhibitory synaptic input (see below).
The increase in excitatory synapse number and decrease in inhibitory synapse number may also result from differential stabilization of synaptic connections and/or upregulation or downregulation of components involved in synaptic maturation. One may consider the following possibilities.

Differential adhesion. The function of synaptic adhesion molecules may be strengthened or impaired by BDNF expression. Interesting pairs of synaptic adhesion molecules can be formed by members of the group of neurexins and neuroligins. Neuroligins are postsynaptic transmembrane proteins which, at the excitatory synapse, bind postsynaptically, to PSD-95, a scaffolding protein, and presynaptically, to β-neurexins which are presynaptic transmembrane proteins. Expression of neuroligins in non-neuronal cells clustered synaptic vesicles in contacting glutamatergic axons thus indicating that neurexin-neuroligin complex functions as a trans-synaptic bridge that aligns neurotransmitter vesicles with the postsynaptic density (Scheiffele et al., 2000). Downregulation of particular isoforms of neuroligins resulted in the preferential loss of excitatory or inhibitory synapses (Chih et al., 2005). These experiments did not address the possible differential action of BDNF on these cell adhesion molecules, but established an experimental model and data base on which such tests could be performed in the near future. The hypothesis to be tested is that BDNF maintains synapse stability by altering the expression and/or function of synapse-type-specific cell adhesion molecules.

Differential effects on neurotransmitter release. NGF and BDNF are reported to facilitate glutamate release (Sala et al., 1998; Canas et al., 2004) while the effects on synaptic GABA release have remained controversial. Recent studies revealed that BDNF elevated the presynaptic levels of glutamic acid decarboxylase 65 (Henneberger et al., 2005) and enhanced the GABA release probability (Baldelli et al., 2005). But inhibition of GABA release by BDNF was also reported (Canas et al., 2004). But one should note that added BDNF decreased the levels of both VGluT2 (see RESULTS) and VIAAT expression (present study and Henneberger et al., 2005). However, an altered mRNA expression must not necessarily lead to a similar change in the level of the respective protein. It has been suggested that

BDNF may assist in the clustering of the VGluT1/2 protein to the synaptic sites. In any case, the higher number of glutamatergic synaptic terminals correlates with the increase in the relative incidence of the miniature excitatory postsynaptic currents (mEPSCs) recorded from the BDNF::EGFP expressing postsynaptic neuron (Fig. 24). Thus, the finding of an increased E/I ratio at the immunocytochemical level is matched by a similar change in transmitter release.

*Differential effects on receptor clustering. A*nother possible explanation of the differential action of BDNF on E and I synapses is that the excitatory and inhibitory receptor clustering could be differentially enhanced or differentially suppressed by BDNF, provided that the frequently assumed link between postsynaptic receptor activity and terminal stabilization indeed exists. In a very elegant study, Elmariah and coworkers showed that in dissociated hippocampal neurons, BDNF treatment not only increased the number and size of NMDARs but also promoted the synaptic localization of these receptors (Elmariah et al., 2004). But these effects required an exposure time of at least 36 h. At inhibitory synapses, BDNF decreased $GABA_AR$ clustering after 12 h (Brunig et al., 2001) and affected the phosphorylation state and cell surface stability of the $GABA_ARs$ at a rapid time scale of minutes (Jovanovic et al., 2004). From the present study, the results show that the differential TrkB-dependent effects of postsynaptically released BDNF on excitatory or inhibitory synapses were present both after 16 and 72 h. A summarizing cartoon illustrates the various routes and mechanisms underlying the BDNF effects on dendrite morphology and synapse formation (Fig. 25).

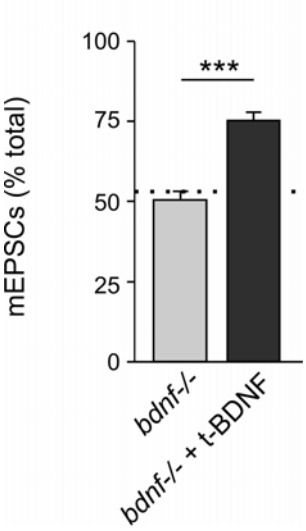


Figure 24. The presence of postsynaptic t-BDNF is associated with an increased fraction of glutamatergic mPSCs. mPSCs were recorded from cultured hippocampal neurons in *bdnf-/-* hippocampal cultures at DIV8. The controls lacked BDNF and were either not transfected (n=11) or transfected with EGFP (n=18). The test neurons (n=14) were transfected with BDNF::EGFP (t-BDNF). Control values obtained from non-transfected *bdnf-/-* neurons are indicated by the dashed line. BDNF-expressing neurons display an increased fraction of glutamatergic mPSCs (frequency of mEPSC / mPSC frequency). From Singh, et al., manuscript in preparation.

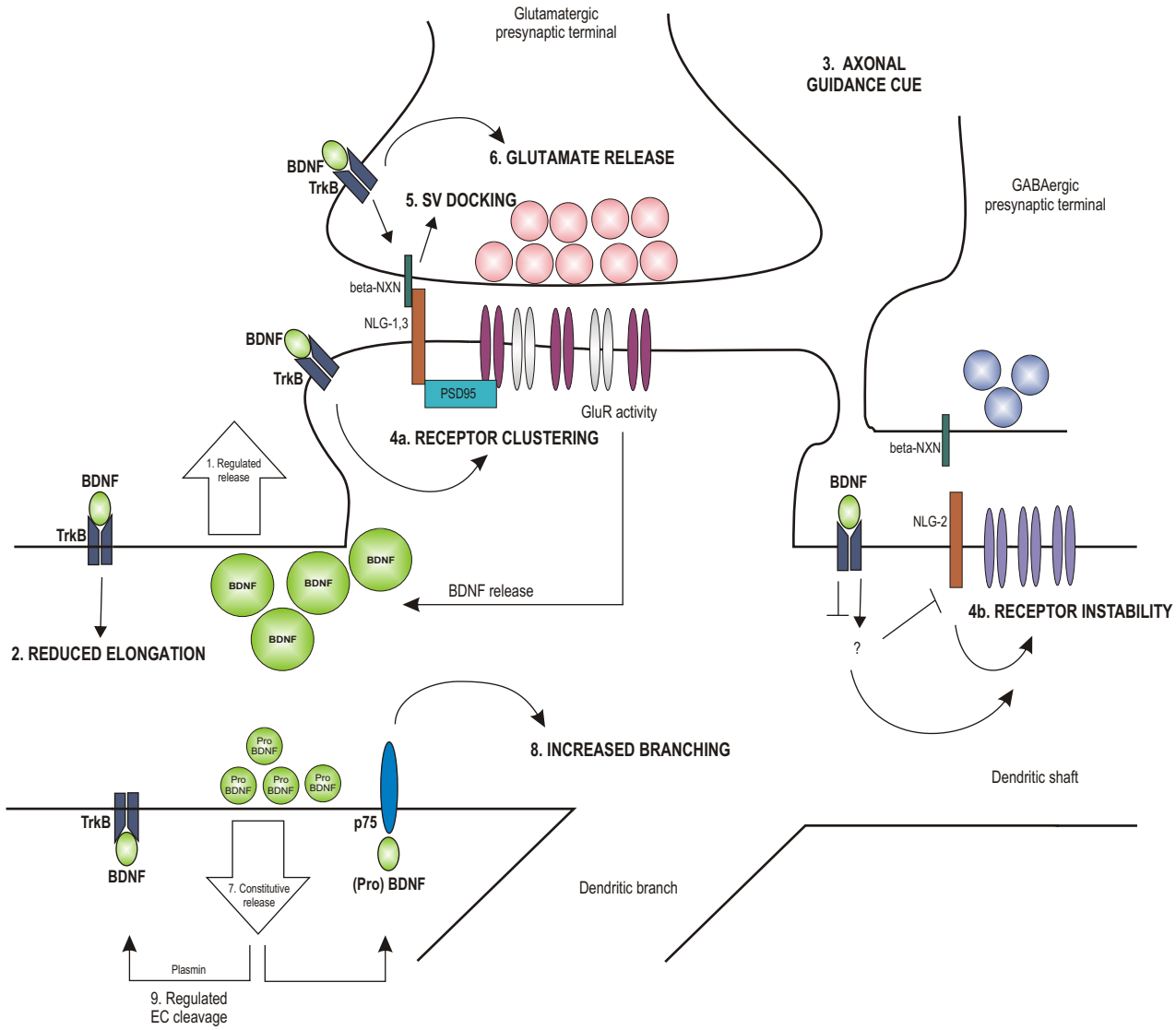
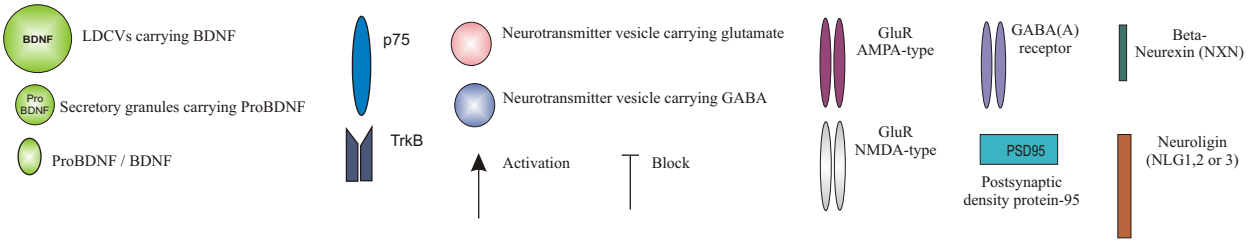


Figure 25. A schematic diagram to illustrate the various routes followed by BDNF in mediating its effects on dendrite morphology and synapse formation. BDNF can be released via two different pathways. **(1)** GluR activity regulated secretion of mature form of BDNF from the large dense core vesicles (LDCVs). **(2)** Mature BDNF binding to postsynaptic TrkB reduces dendritic elongation. **(3)** Mature BDNF can act as an axonal guidance cue serving as a chemoattractive signal for glutamatergic and chemorepulsive signal for GABAergic presynaptic temrinals. **(4)** Mature BDNF may regulate receptor clustering thus stabilizing **(4a)** or destabilizing **(4b)** the glutamatergic or GABAergic synaptic terminals respectively. **(5)** BDNF binding to presynaptic TrkB may enhance synaptic vesicle (SV) docking. **(6)** It can also enhance glutamate release. **(7)** Second pathway for secretion of BDNF is the constitutive pathway by which BDNF is majorly secreted in the precursor form (ProBDNF). **(8)** ProBDNF or mature BDNF may bind to p75 to increase dendritic branching. **(9)** Extracellular (EC) cleavage of ProBDNF by plasmins secreted at synaptic sites may be regulated by GluR activity so that TrkB related effects of BDNF are linked to GluR activation.



3.2. Possible consequences of increased E/I ratio of synaptic terminal numbers

If also present during normal development in the brain, a BDNF-induced alteration of the balance between excitatory and inhibitory synapse formation may have consequences for the activity and survival of hippocampal neurons. The BDNF-induced shift of the E/I balance of synaptic input toward will inevitably produce enhanced network activity which may be relevant to the etiology of neurological disorders with synaptic dysfunction such as epilepsy, schizophrenia and autism (Jamain et al., 2003). Anatomical studies in human tissue from temporal lobe epileptics showed increased mRNA for BDNF in granule cells (Mathern et al., 1997). Spontaneous seizures in transgenic mice overexpressing BDNF (Croll et al., 1999) and hyperexcitability of the hippocampus in rats treated with BDNF (Scharfman, 1997) imply that higher than normal levels of BDNF represent a risk for increased neuronal damage or death. Thus, endogenous BDNF may be required for normal brain development, but higher levels may have adverse effects. Transfection of BDNF::EGFP into neurons originally lacking BDNF provides a very strong stimulus to the neurons which may result in particularly drastic responses. If we examine the E/I ratio of synaptic input, we observe that within 72 h of BDNF expression becomes relatively moderate, but still remain nearly double. Therefore, from the present experiments, it seems that at least in the hippocampus, a critical level of BDNF and TrkB activation is necessary for modulating synaptogenesis as well as dendrite growth. Therefore, a regulated release or function of BDNF seems utterly important.

In view of the clinical relevance of a possible BDNF-induced excitotoxicity, it was asked whether chronic absence or presence of BDNF in neurons dissociated from hippocampus of *bdnf-/-* mice, affected neuronal survival. Several reports have suggested that added neurotrophins increase the viability of hippocampal and cortical neurons (Finkbeiner, 2000). The present analysis of nuclear staining in hippocampal neurons showed no effect of the addition of neurotrophins (NGF, BDNF and NT-3) on the survival of hippocampal neurons.

There was also no difference in the survival rate of cultured neurons obtained from *bdnf+/+* as opposed to those from *bdnf-/-* mice. However, neurons expressing BDNF::EGFP in *bdnf-/-* cultures tended to less viable, as deduced from the lower incidence of the EGFP-positive neurons in coverslips transfected with BDNF::EGFP as opposed to those transfected with EGFP alone.

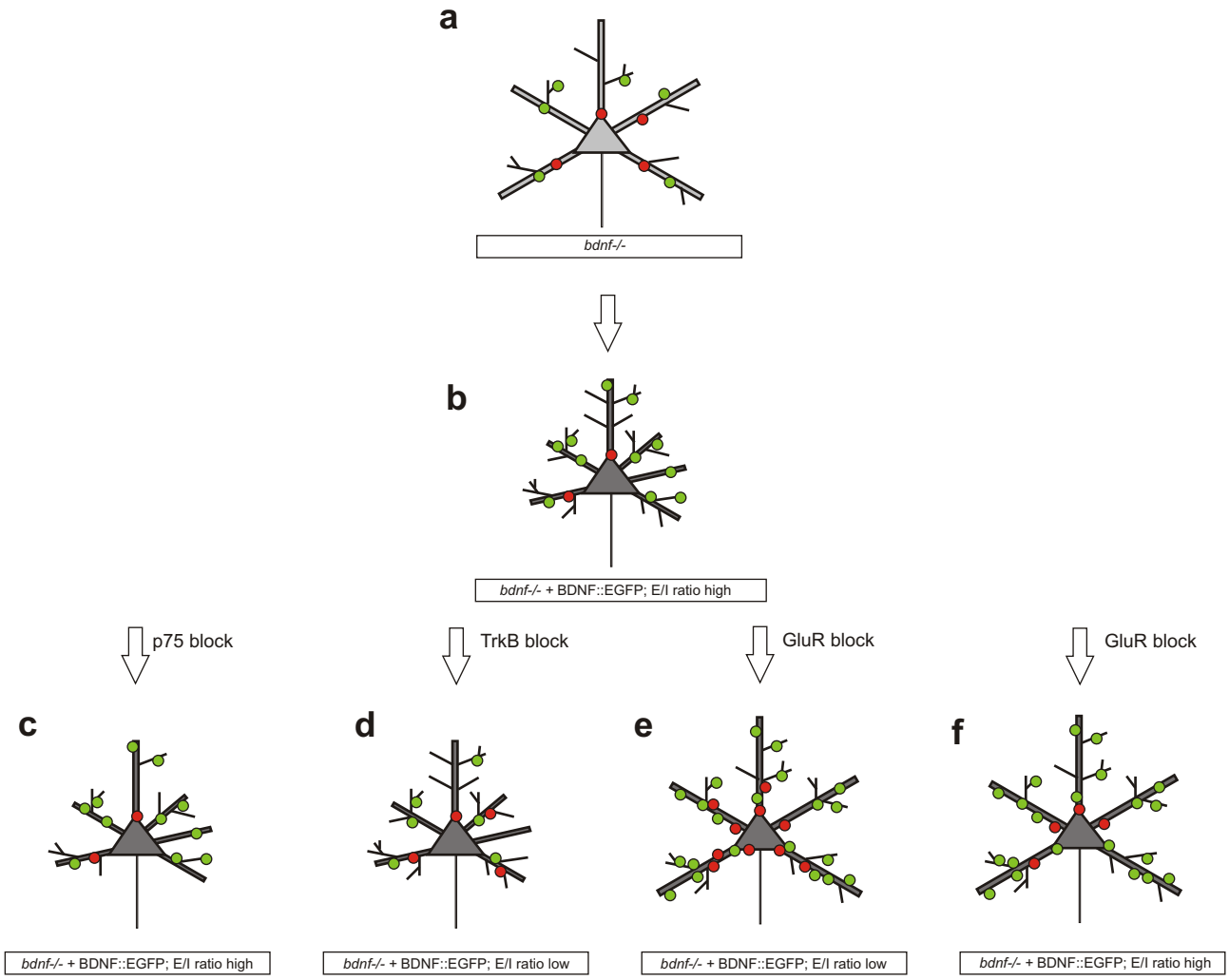


Figure 26. A schematic representation of the effects of transfection of BDNF::EGFP in *bdnf-/-* neurons. (a) A *bdnf-/-* neuron with its dendritic structure and the glutamatergic synapses (green dots) and GABAergic synapses (red dots). **(b)** The dendrite morphology and the excitatory-to-inibitory (E/I) ratio of glutamatergic to GABAergic synaptic terminals is changed drastically after 16 h of BDNF::EGFP expression. A BDNF::EGFP- transfected neuron displays a larger number of primary dendrites but with a shorter length and more branch points.The E/I ratio shifts toward higher E in BDNF::EGFP-transfected neurons. **(c-f)** This ratio is unaffected after p75 block (c) but is altered after TrkB block (d). Block of glutamate receptor (GluR) activity may result in two possibilities, first is shown in (e) that the increase in E-type synaptic terminals is parallel to an increase in I-type synaptic temrinals keeping the E/I ratio constant (as low as in *bdnf-/-*). The second possibility is depicted in (f) where I-type synaptic terminal number is unaffected thus keeping the E/I ratio high (as in t-BDNF).

We considered the possibility that the drastic increase of the E/I ratio would result in stronger depolarization due to E predominance. But, block of GluRs during the expression period could not prevent the lower incidence of BDNF::EGFP-positive neurons. Thus, other reasons must account for the reduced survival rate of BDNF-expressing neurons in these preparations.

4. ACTIVITY-DEPENDENCY OF DENDRITE MORPHOLOGY AND SYNAPTOGENESIS

4.1. Receptor-specific control of dendrite branching and initiation by BDNF

The present experiments revealed a negative GluR-dependent effect of BDNF on dendrite length which is consistent with a similar action in retinal ganglion cells (Lom and Cohen-Cory, 1999) although, in general, very little is known on the relationship between synaptic activity and dendrite growth. A recent study reported that added BDNF affected dendrite growth in cortical neurons via phosphoinositidyl-kinase (PI3-K) and mitogen activated protein kinase (MAP-K) pathways (Dijkhuizen and Ghosh, 2005), both of which are activated through Trk receptors. Our results together with this study suggest that these two signaling pathways link BDNF receptor activation to dendrite growth in developing neurons. It is expected that PI3-K pathway regulates dendritic development by directly regulating cytoskeletal proteins by controlling the activity of some Rho family GTPases. Rho GTPases have a principal role in mediating dendrite growth and remodeling in developing neurons (Threadgill et al., 1997; Luo, 2000). Activation of Rho GTPases is facilitated by guanosine exchange factors (GEFs) that switch Rho family GTPases from an inactive GDP-bound state to an active GTP-bound state (Kjoller and Hall, 1999). The activity of many GEFs can be regulated by PI3-K activation, providing a possible link between PI3-K pathway and dendrite growth through Rho family GTPases (Han et al., 1998). Another important mechanism implicated in dendrite growth is the regulation of microtubule dynamics followed by activation of the MAP-K pathway (Reszka et al., 1997). NGF-mediated activation of MAP-K pathway and subsequent phosphorylation of MAP2 were recently shown to support dendrite development of sensory neurons (Vaillant et al., 2002) and sympathetic neurons (Kim et al., 2004). Therefore, involvement of specific neurotrophin receptors for parallel activation of PI3-K and MAP-K pathways may be necessary to regulate actin and microtubule dynamics, respectively.

Another interesting outcome of the present study was that p75, but not TrkB, mediated the enhanced branching of hippocampal neurons under the influence of BDNF. This reflects the complexity of the neurotrophic control of dendrite growth. So far, p75-mediated NT effects have been studied in axons. It has been shown that p75 null mutant mice displayed stunted growth and reduced arborization (Bentley and Lee, 2000). However, in a recent study on hippocampal neurons, p75 activation was shown to participate in the regulation of dendrite morphology by NGF (Salama-Cohen et al., 2005). Consistent with these reports, the present experiments support a p75-related pathway in dendrite development, but more specifically in dendritic branching.

4.2. Effects of glutamate receptor blockers on BDNF-induced changes of dendrite morphology

As it is known that synaptic activity can play a role in dendritic growth, the possibility that in BDNF-expressing cells, the dendrites respond to the increased excitatory synaptic input was considered. This could best be tested by performing experiments with blockers of glutamate receptor activity and in the presence or absence of a TrkB blocking agent. The data from experiments using a mixture of glutamate receptor antagonists showed that activity block opposed the action of BDNF with regard to dendrite elongation. Thus, GluR block had an effect similar to TrkB blockade. However, a strong potentiation in the number of glutamatergic synapses was seen in BDNF::EGFP transfected neurons in presence of GluR blockers. This kind of response appeared to be operating via a compensatory feedback loop, where loss of activity induces formation of more synapses. This phenomenon was seen even in the absence of BDNF. But despite the even higher number of synapses BDNF-induced dendrite elongation was prevented by the absence of receptor activity. Therefore, we can conclude that the BDNF-induced suppression of dendrite elongation was not a consequence of enhanced contact number as such, but it reflected the enhanced activation of glutamate receptors on the BDNF-expressing dendrites.

It has been proposed that synaptic activity may cause calcium influx via transmitter and voltage-gated calcium channels, which would cause a local increase in intracellular calcium concentration. Calcium triggers a variety of signal transduction pathways involving Rho GTPases, MEK/ERK, and CaMKII, which may ultimately initiate changes in the dendritic

cytoskeleton. Previous work in cultured slices from ferret visual cortex has also shown that blocking glutamate receptors or L-type calcium channels increased dendrite growth in pyramidal and non-pyramidal neurons (McAllister et al., 1996; Baker et al., 1997).

In contrast to dendrite length, dendrite branching was not correlated with glutamatergic synapse number nor was it sensitive to TrkB or GluR block. It was therefore, concluded that the positive relationship between the number of branch points and the number of glutamatergic synaptic terminals reflected a correlation rather than a causal relationship.

5. ROLE OF PRG-1 IN SYNAPTOGENESIS

Considering the upregulation of PRG-1 in the entorhinal cortex after lesion of the perforant path (Savaskan, unpublished results), the present experiments with overexpression of PRG-1 in dissociated, cultured neurons from the entorhinal cortex could serve as a model to test for a role of PRG-1 in guarding axotomized neurons against synaptic stripping. The evaluation showed that PRG-1 prevented the LPA-mediated reduction in the number of glutamatergic synaptic terminals. Suppression of the PRG-1 gene resulted in a reduction of glutamatergic synapse number even in the absence of LPA. It was also found that a reduction in the number of GABAergic terminals occurs after PRG-1 gene silencing, but this was only seen after LPA-challenge. From these results, we concluded that PRG-1 could be involved in synapse formation and/or stabilization of glutamatergic synapses. Thus, under LPA-challenge, PRG-1 could also assist the stabilization of GABAergic synapses.

5.1. Preferential formation or stabilization of excitatory synapses in dependence on PRG-1

The present experiments suggest that PRG-1 is essential for the formation and/or stability of excitatory synapses. Moreover, a similar positive effect of PRG-1 was observed in case of GABAergic synapses as well, but only in the presence of LPA-challenge. The results can tentatively be interpreted by assuming two distinct functions of PRG-1. In the anterograde direction it may be necessary for the axon to form synapses in the target, as already suggested by Bräuer and colleagues. In the retrograde direction it may be necessary for the dendrites to maintain the synapses in a defense situation, as is given after axotomy. The latter role could be tested by LPA-treatment. Thus, considering a general expression of PRG-1 throughout the

neuronal plasma membrane, it seems possible that PRG-1 interacts, at selected sites, with different molecular components to form or to stabilize synapses. The downstream molecular partners of PRG-1 are still unknown, but from our experiments, it is expected that, if LPA binds to PRG-1, then the LPA-bound and unbound forms of PRG-1 can exist in two different (conformational/active) states which confer it a capacity to bind/activate different interacting partners at the two kinds of synaptic terminals. The long intracellular C-terminal domain of PRG-1 may be involved in these mechanisms. PRG-1 could also serve as a plasma membrane receptor (for LPA or other unidentified ligands) to activate yet unidentified regulatory or signal transduction pathways.

5.2. Possible mechanism of action of PRG-1

Amino acid sequence comparisons have revealed some interesting similarities as well as dissimilarities among all the lipid phosphate phosphatases (LPPs) and lipid phosphate phosphatase-related proteins (LPRs/PRGs). The LPPs possess three conserved active site domains (domains 1, 2 and 3) and six transmembrane domains. There are some conserved amino acid residues in these domains which are shown to be essential for the ectophosphatase activity of LPPs which convert phosphatidic acid to diacyl glycerol and inorganic phosphate (Brindley, 2004; Zhang et al, 2000). The PRGs lack the conserved lysine and arginine residues in domain 1 and the histidine in domain 3. However, the domain 2 of PRG-1 carries a conserved arginine and histidine which can explain for its activity as an ectophosphatase despite the lack of other conserved residues which are shown to be essential for this function. Moreover, there are additional non-conservative substitutions of the domain 2 motif residues. Thus, this incomplete conservation of the catalytic motif in the PRG proteins implies that PRG-1 utilizes a mechanism to catalyze lipid phosphatase reaction, which is different from that shown by the members of the LPP family which involve their domains 1 and -3. Another possibility is that PRGs do not participate in the dephosphorylation reaction but rather indirectly stimulate the activity of other existing LPPs. But, this requires to be confirmed by mutating or gene silencing of the LPPs. In contrast to PRG-1, the expression of another member of the PRG family, the PRG-3 did not increase LPA dephosphorylation activity (Savaskan et al., 2004). Overexpression of PRG-3 in neurons and COS-7 cells promoted neurite extension and cell spreading. Even in the absence of ectophosphatase activity, PRG-3

was able to mediate these morphological effects. Hence, there should exist another mechanism which is independent of LPA signaling, through which the PRGs could have an effect on cell structure and cell signaling. This is supported by our results on silencing of gene expression of PRG-1. However, confirmatory experiments need to be performed, for example, using transfection with a control siRNA, or constructs consisting of mutations in the presumptive active domains of PRG-1.

5.3. A role for PRG-1 in dendritogenesis?

Our experiments showed that overexpression of PRG-1 also prevented the mild LPA-induced increase (+25.8± 5.3%) in dendritic branching. But, by itself, there appeared to be no direct effect of PRG-1 on dendrite morphology. Therefore, we explain the effect on branching as an indirect consequence of LPA deactivation. One could also argue that PRG-1 helps to stabilize the already existing dendritic branches in line with the synaptotropic hypothesis. Interactions between other synapse stabilizing molecules like N-cadherins and beta-catenin have been implicated. Therefore, a downstream convergence of signaling pathways mediated by these CAMs and PRG-1 seems quite likely. For example, NCAM mediates activation of PKC through PLC-γ associated signaling which may regulate neurite outgrowth and synapse assembly (Kolkova, 2005; Polo-Parada et al., 2004). However, more experiments are needed to finally understand the role of PRG-1 in dendrite development. The availability of a PRG-1 mutant mice would allow for more specific experiments in order to clarify the function of PRG-1 in dendrite development and synaptogenesis.

5.4. Possible functions for PRG-1

Our experiments provided first evidence for a role for PRG-1 in synapse stabilization and dendrite development. But additional and yet unknown functions of PRG-1 can also be envisaged because of some unusual structural features of this protein. For instance, the cytoplasmic C-terminus hydrophilic tail which is about 400 aa long, is highly enriched in charged amino acid residues and contains PEST [proline (P), glutamic acid (E), serine (S) and threonine (T)-containing] sequences. The PEST sequences in other proteins have been shown to serve as signals for ubiquitin-dependent proteolytic degradation (Rechsteiner and Rogers, 1996). In the mouse PRG-1, the sequence 537 HIPEETQENISTSPK 551 and 707

RVTPVEGSEIGSETLSVSSSR 727 are potential PEST sequences. From one point of view, the difference in activity of PRG-1 in presence or absence of LPA could be explained by the fact that, on binding to LPA, a conformational change in PRG-1 protein structure could mask or unmask the PEST sequences. If this is a case, then LPA could serve as a conditional proteolytic signal or even a ligand controlling the metabolic stability of PRG-1. Therefore, proteolytic degradation may be another mechanism for regulation of the turnover and expression levels of the PRG-1. On comparing the expression of the various PRGs, a non-overlapping expression pattern of the PRG genes was found. This suggests that, in contrast to the LPPs which show a similar and overlapping expression pattern in various tissues, the PRGs have a non-redundant biological function associated with them. Considering the intracellular distribution of PRG-1 as shown by an ultrastructural analysis of the outer molecular layer of the hippocampus which is the terminal zone of the axons originating from the entorhinal cortex, PRG-1 immunoreactivity specifically in the growth cone-like specializations was seen (Bräuer et al., 2003). But, we observed a rather diffuse distribution of EGFP-tagged PRG-1 in the soma, dendritic tree as well as the axons of hippocampal neurons. So, there remain many intriguing issues with regard to the subcellular expression pattern of PRG-1, as well as its signaling mechanisms, which need to be addressed ahead.

CONCLUSIONS

From this study, the following conclusions can be drawn.

1. Low efficiency transfection of neurons in a BDNF-deficient environment (*bdnf-/-*) is a suitable experimental approach that allows to clarify local effects of BDNF on dendrites and synapses

The low efficiency of BDNF::EGFP transfection in *bdnf-/-* neurons allowed to characterize the actions of a local cellular source of BDNF in a large number yet totally isolated hippocampal neurons *in vitro*. Another advantage of this approach is that one can distinguish between the effects of proteins acting from the pre- or postsynaptic side. Our transfection model was also an appropriate system to identify autocrine as opposed to retrograde effects of BDNF.

2. A local cellular source of BDNF is more effective than diffusely distributed exogenous BDNF

A neuronal source of BDNF can modify dendritic morphology and synaptogenesis more effectively than added BDNF. Although the changes seen in both the cases were similar, the effects produced by transfected BDNF were higher in magnitude underlining the significance of BDNF gradients and activity-dependent release at synaptic sites. The locally concentrated extracellular BDNF released by the transfected neurons, follows an autocrine and a retrograde route to influence dendrite morphology and synapse formation. It inhibits dendrite elongation and promotes synaptogenesis via TrkB signaling and enhances dendritic branching via p75 signaling.

3. BDNF produces a strong increase in the total number of synaptic terminals and shifts the E/I ratio of synaptic input

The presence of BDNF in a postsynaptic neuron is a strong attractant or stabilizing factor for BDNF-deficient afferents. In addition, it was found that BDNF alters the balance of synaptic inputs in single neurons by upregulation of glutamatergic excitatory (E) and downregulation

of GABAergic inhibitory (I) synaptic contacts formed at transfected hippocampal neurons in culture. The downregulation of I input might be a consequence of the stronger E input, as both required TrkB activity.

4. BDNF transfection changes dendritic morphology. The BDNF-induced suppression of dendrite elongation is mediated by TrkB and GluR activity, while the enhancement in dendritic branching is mediated by p75

BDNF-transfected neurons display less dendrite elongation but enhanced outgrowth of dendritic branches. These effects require the activity of different NT receptors. While block of TrkB abolishes the former, the latter effect is more sensitive to block of p75. This indicates that two different signaling pathways of BDNF may account for the control of dendrite morphology. However, the suppression of dendrite elongation appears to be an indirect effect that merely reflects the upregulation of glutamatergic synaptic function, because block of glutamate receptor activity abolishes this effect despite a higher number of glutamatergic contacts.

5. PRG-1 can contribute to the selective stabilization of excitatory synaptic contacts

Block of PRG-1 expression by transfection with siRNA against PRG-1 produces a strong decrease in the number of synaptic contacts at the hippocampal or entorhinal cortical neurons. PRG-1 can also protect these neurons against a challenge with LPA which would cause a drop in the number of glutamatergic synaptic terminals in the absence of PRG-1 overexpression. These data suggest that PRG-1 can be involved in synapse stabilization, notably under condition of axotomy or other factors leading to enhanced synapse elimination.

Prospects

In this study, postsynaptic BDNF and PRG-1 proved to be essential factors in synapse development. To make further progress, it would be of much importance to devise a method for inducing and quantifying regulated release of BDNF from transfected neurons to study acute BDNF in live cells during synaptogenesis or synapse elimination. Also very interesting would be to look for eventually existing synergistic interactions between BDNF and PRG-1 in synapse formation, or to find out if BDNF can compensate for a loss of PRG-1.