RESULTS

1. EXPERIMENTAL MODEL

1.1. Selection of tissue and cells

Hippocampal neurons were chosen for the major part of this study, for the following reasons. First, BDNF has the highest level of expression in the hippocampus and its high affinity receptor, TrkB is also found in high abundance in this region (Ernfors et al., 1990a, 1990b, 1991; Hofer et al., 1990). Second, as most of the studies on the modulation of synaptic transmission by neurotrophins or other factors have been performed in the hippocampus (Kang and Schuman, 1995, 1996; Figurov et al., 1996; Korte et al., 1995; Costa-Mattioli et al., 2005), the experiments in the present study would complement those concepts by providing new data on synapse formation during the early stages of hippocampal development. Third, the hippocampus is source of a relatively homogeneous population of neurons. The majority (85-90%) of neurons in culture develop into the pyramidal phenotype, the principal cell class in the hippocampus (Goslin et al., 1998) which has a well characterized morphology that offers a reasonable possibility to link BDNF- or PRG-1-induced changes in dendrite morphology and synapse development to those *in situ*.

1.2. Cell culture and transfection paradigm

To answer our question on the role of BDNF and PRG-1 in synapse formation and dendrite morphology during development, we made use of a low density primary cell culture system derived from the mouse hippocampus (Goslin et al., 1998). The age selected for plating the neurons was E18. The final neuron density in the evaluated cultures was 60,000/ cm^2. For the experiments described in chapters 2-4, the BDNF-deficient neurons were allowed to develop in culture for a period of 5 days, after which, on DIV 5, a very small fraction of neurons (0.02%) was transfected with EGFP or EGFP-tagged BDNF (BDNF::EGFP). To establish a local source of BDNF, the gene expression was limited to 16 h (or on few occasions, 72 h), after which the neurons were fixed and subjected to evaluation. Only non-overlapping EGFP-labeled neurons (average distance from the next labeled soma was 2.2 mm) in cultures

displaying uniform density were included for further analysis. Experiments with EGFP-tagged PRG-1 (PRG1::EGFP) transfection were carried out in the same way, except that the primary neuronal cultures were prepared from wild-type C57/BL/6 mice. So, the latter was an overexpression study. The neuron age corresponded to a postnatal stage when synaptogenesis is very active in the developing hippocampus (Super and Soriano, 1994). In these transient transfection systems, expression of the reporter gene EGFP in primary neurons is not likely to produce toxicity, as a significant fraction of cells undergoing cell death was not detected from the DAPI staining which is commonly used for this purpose. Induction of apoptosis in non-neuronal cell lines expressing EGFP has been reported, but only after several days of expression (Liu et al., 1999). EGFP can also produce toxicity by generation of free radicals on long term excitation. Our analysis involved excitation mostly on fixed preparations, therefore, any cell death occurring in the dissociated cultures may not be attributable to EGFP transfection.

1.3. Quantification of neuronal survival

Addition of neurotrophins is expected to improve the trophic status of the cells in culture and could support survival in addition to dendrite growth and synapse formation. Hence, in order to test for any effect on neuron survival, the neuron density was compared in the view fields, in absence and presence of exogenous neurotrophins, BDNF, NGF or NT-3 and also in cultures containing EGFP- as opposed to BDNF::EGFP-expressing neurons. Healthy neurons were identified by nuclear staining with DAPI (4`, 6 diamidino-2-phenylindole; Fig. 3).

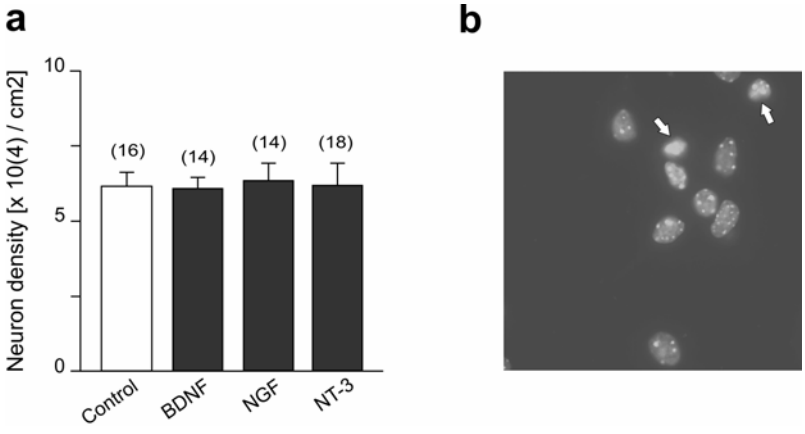

Figure 3. Exogenous neurotrophins did not affect neuron survival. (a) Quantification of neuronal density. Addition of BDNF, NGF or NT-3 in the culture medium did not alter the neuronal density

after 6 days in culture. The cells were plated at an equal plating density and found to be comparable for all the treatment groups and the untreated controls (60,000 cells/cm^2). **(b)** Healthy neurons were identified by nuclear staining with DAPI (4`, 6 diamidino-2-phenylindole). Primary hippocampal neuron cultures were fixed and subjected to nuclear chromatin staining with DAPI. Note large round healthy nuclei with undistorted nuclear membrane. Pycnotic nuclei as visible by their shrunken and condensed appearance (arrows) were excluded from evaluation.

DAPI is excited at 360 nm which produces a blue fluorescence at about 460 nm, when bound to DNA. Pycnotic nuclei are characterized by their fragmented and shrunken appearance which is suggestive of cell damage. Therefore, healthy neurons were expected to have a single round nucleus with unfragmented nuclear membrane (Laudenbach et al., 2002). We found that the survival assessed by the average number of neurons per view field was not affected by any treatment condition. There were no differences between the groups, confirming that the doses of exogenous neurotrophins did not enhance survival and not affect dendritic growth or synapse formation by increased trophic support. The survival of *bdnf*-/- neurons in culture also did not differ from the *bdnf*+/+ neurons.

1.4. Parameters studied

For any given neuron, we studied the following parameters: 1) The number of primary dendrites present in the neuron, i.e., the first order dendrites which projected directly from the neuronal soma. 2) The number of branch points within a circular view field (ROI) of 50 µm radius from the soma center. A branch point was defined as the point of origin of at least two daughter branches, each exhibiting a length of at least 10 µm. 3) The number of dendritic trees reaching or extending beyond the margins of the view field. This number was used to assess the dendritic elongation. The total length of the entire dendritic arbor was not measured because an expected change in branching would have influenced any existing difference in the total length of the dendritic trees. 4) The fraction of the dendritic trees reaching or exceeding the view field. 5) The number of synaptic terminals contacting the labeled neuron within the view field. The evaluation of the dendrite morphology and synapse formation was done by manual counting of the above-mentioned parameters.

1.5. Identification of dendrites

In a sub-set of experiments, the dendrites were identified by their positive staining for microtubule-associated protein2 (MAP2), a specific dendritic marker (Caceres et al., 1984). Neurons transfected with EGFP, BDNF::EGFP or PRG1::EGFP were fixed and subjected to double immunocytochemical staining for EGFP and MAP2. Neurites which displayed co-localized immunoreactivity for both EGFP and MAP2 were classed as dendrites. Processes less than 10 µm in length (Lom and Cohen-Cory, 1999) or lacking MAP2 immunoreactivity or both, were not considered as dendrites. The axons showed EGFP immunoreactivity but stained negative for MAP2. Only those neurons which possessed a well defined axon were considered for this study. Based on these double staining experiments, we established the following criteria to exclude axons from evaluation in all following experiments where MAP2 staining was not performed. 1) At its origin, the diameter of the axon is smaller than that of a dendritic shaft and it remains constant, unlike dendrites which have a diameter tapering along the length of the dendritic tree. 2) Axons give rise to collaterals at more or less right angles to the main branch while dendrites bifurcate at acute angles. 3) The length of an axon is at least thrice that of the largest dendritic tree of the same neuron and typically can be traced up to hundreds of micrometers, a distance far exceeding the dendritic field (Dotti et al., 1988).

1.6 Definition of synaptic terminals

Synaptic terminals in contact with the dendritic surface of an EGFP-labeled neuron were identified by single immunofluorescence using an antibody against synaptophysin I (Syp I), a conventional presynaptic marker (Jahn et al., 1985; Fletcher et al., 1991). In order to distinguish glutamatergic synaptic terminals from GABAergic synaptic terminals, triple labeling was performed applying antibodies against one of the transmitter-selective vesicular transporters (vesicular glutamate transporters 1 and 2, VGluT1/2, or vesicular inhibitory amino acid transporter, VIAAT) and, in most of the experiments, one additional synaptic marker such as Syp I. Colocalized terminals positive for VGluT1/2 or VIAAT and Syp I were classified as glutamatergic and GABAergic synaptic terminals, respectively. To avoid false positive/negative counts, we also used a size criterion to validate the counts. Assuming a more or less round or elliptical shape of the contact, a counted putative terminal was observed to have suprathreshold intensity values over an area of at least 0.2 μm^2, the threshold intensity

was typically equal to the average intensity of the ROI plus three times its standard deviation. An in-house software (NeMo) developed by Dr. Henneberger in our lab, was used for this test, in some of the experiments.

2. ACTION OF EXOGENOUS NEUROTROPHINS (BDNF, NGF AND NT-3)

2.1. Comparison of the effects of BDNF, NGF and NT-3.

First the effects of long term exposure to BDNF and other neurotrophins (NTs) on dendrite morphology and synapse formation at early stages of neuronal development were examined. To this end, the culture medium was continuously supplemented with 100 ng/ml of either BDNF, NGF or NT-3 during the first five days in culture. On DIV 5, the cultures were transfected with EGFP plasmid, and after 16 h of expression time, the cultures were fixed and subjected to double immunocytochemistry applying primary antibodies against EGFP and Syp I. Table 2 summarizes the results.

Parameters studied	**Control n= 60** (Mean ± SEM)	**BDNF n= 52** (Mean ± SEM)	**NGF n= 55** (Mean ± SEM)	**NT-3 n= 56** (Mean ± SEM)
Neuron density [x 10(4)/ cm^2]	6.1 ± 0.4	6.0 ± 0.4	6.3 ± 0.6	6.1± 0.7
Number of primary dendrites	4.3 ± 0.3	4.8 ± 0.3	4.0 ± 0.3	5.2 ± 0.3
Number of dendrites > 50 µm	2.6 ± 0.4	2.7 ± 0.2	2.2 ± 0.1	2.7 ± 0.2
Fraction of dendrites > 50 µm	48.9 ± 4.6%	58.2 ± 4.4%	59.5 ± 3.5%	57.3 ± 4.5%
Number of dendritic branch points per ROI	4.2 ± 0.4	6.7 ± 0.6 ***	5.2 ± 0.6	7.0 ± 0.6 ***
Number of synaptic terminals per ROI	14.0 ± 0.9	18.6 ± 1.6 **	15.8 ± 1.6	13.1 ± 1.6

Table 2. Comparison between added neurotrophins on their effects on dendrite morphology and synapse number after chronic exposure. Primary hippocampal neuronal cultures from wildtype C57/BL/6 mice were treated with either BDNF, NGF or NT-3 at a concentration of 100 ng/ml for a period of 5 days. The number of neurons studied for the respective treatment group is denoted by 'n'. Significance levels are indicated as ** $p<0.01$ and *** $p<0.001$. SEM, standard error of mean; ns, not significant; ROI (50 µm radius, centered at the soma).

Together, the experiments with treatment of primary hippocampal neurons from wild-type mice, with neurotrophins, indicate that unlike NGF and NT-3, BDNF treatment resulted in a marked increase in the number of synapses. It also increased the dendritic branch point number. This result is in line with previous *in vitro* studies which suggested a role of BDNF in the development of neurons and synaptic plasticity (Lu and Figurov, 1997; McAllister et al, 1999; Tyler and Pozzo-Miller, 2001; Rico et al., 2002).

As it is known that long term treatment may attenuate initial short term effects of BDNF, experiments where BDNF was applied for 16 h to cultures developing for an initial 5 DIV in BDNF-deficient environment (*bdnf-/-*) were then performed. The results of these experiments are presented in Fig. 4. It can be seen that the number of primary dendrites increased from a control level of 4.4 ± 0.1 (n=137) to 5.8 ± 0.2 (n=166) in the test (Fig. 4b). The number of branch points also increased from 9.1 ± 0.4 (control) to 12.4 ± 0.4 (treatment with BDNF). No difference was found in the number of dendritic trees exceeding the length of 50 µm. However, the fraction of dendritic trees > 50 µm dropped from 59.9 ± 2.4% in control neurons to 40.5 ± 2.1% in treated neurons. The number of Syp I-positive terminals was increased by the addition of BDNF from 10.3± 0.6 (control) to 14.8 ± 0.7 in BDNF-treated neurons (Fig. 4f). These results indicate that exogenous application of BDNF, notably, if applied for shorter times, stimulates dendrite initiation and synapse formation.

2.2. Exogenous BDNF alters mRNA expression of presynaptic proteins and TrkB

To determine if the increase in synapse numbers is associated with an increase in the expression of proteins required for synapse formation, the mRNA profile of some of the active zone proteins as well as receptor TrkB, in the presence of added BDNF, was analysed. It has been shown earlier by Western blot analysis that BDNF can regulate the expression of synaptic vesicle proteins through a cAMP dependent mechanism (Tartaglia et al., 2001), but, it is not yet clear if the increase in protein expression was associated with an increase in the respective mRNA transcription. Moreover, analysing expression levels of proteins specific to excitatory and inhibitory synapses is of high interest in the light of an expected differential regulation of these two kinds of synapses by BDNF. Therefore, we measured the overall mRNA expression levels of the synaptic proteins, synaptophysin I (Syp I) and synapsin I (Syn I) which are found in both glutamatergic and GABAergic synaptic terminals, as well as

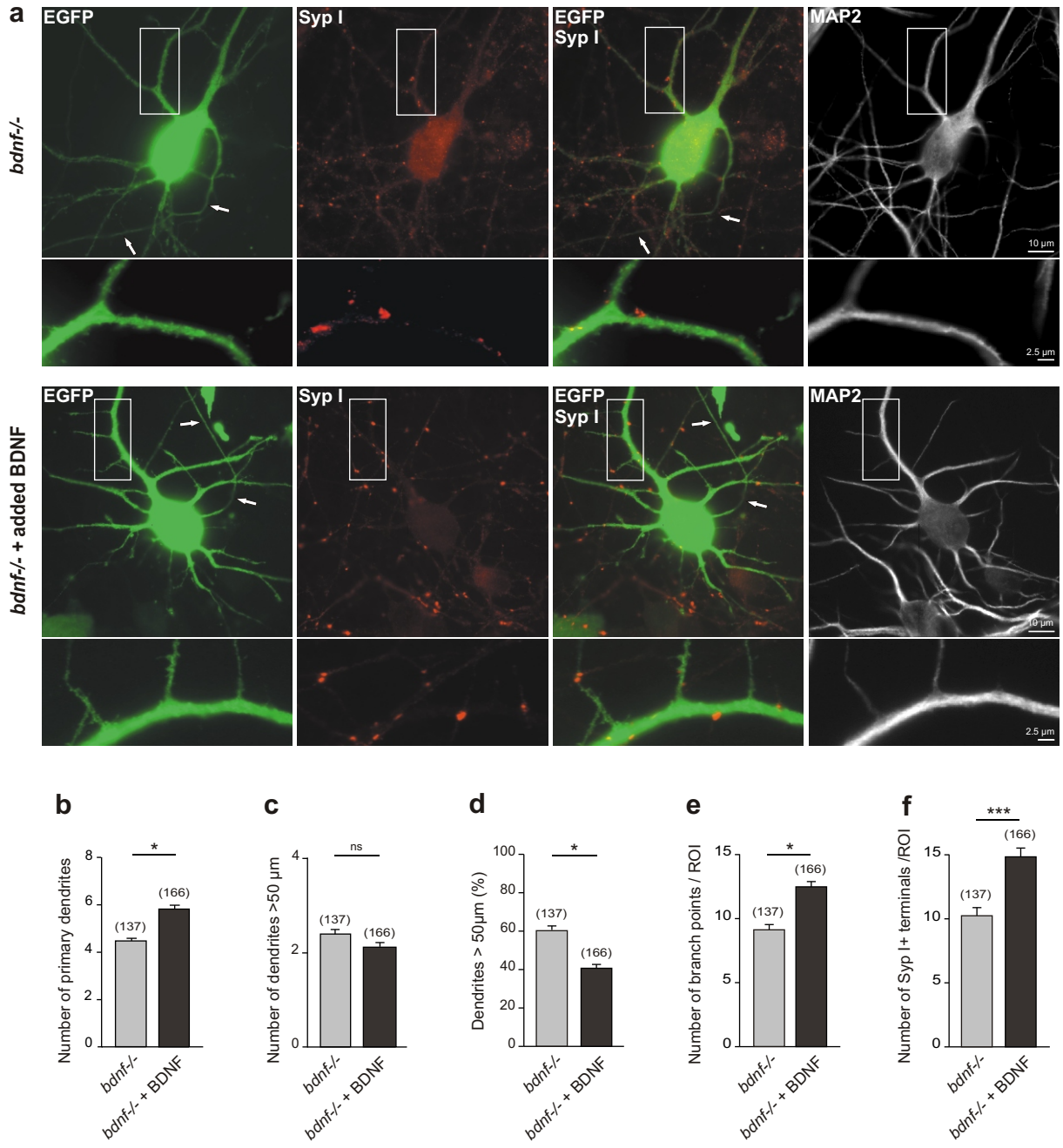

Figure 4. Exposure to exogenous BDNF stimulates primary dendrite formation, dendrite branching and synapse formation but reduces dendrite elongation. (a) Images of EGFP expressing *bdnf-/-* neurons in primary hippocampal dissociated culture with (lower panel) or without (upper panel) 16 h treatment with exogenous addition of BDNF (100 ng/ml). On DIV 5, neurons were transfected with EGFP and allowed to express the plasmid for 16 h in BDNF-supplied medium. Triple immunocytochemistry was performed with antibodies against EGFP (pseudocolored as green), synaptophysin I (Syp I, pseudocolored as red) and a dendritic marker, microtubule-associated protein, MAP2 (black and white). Dendrites were distinguished from axons (arrows) by their selective staining for MAP2. Arrows indicate the axons which lack MAP2 immunoreactivity. **(b-f)** Quantification of the short-term effects of added BDNF on primary dendrite formation (b), dendrite elongation, as assessed by the number and percent fraction of dendritic trees > 50 µm (c and d respectively), number of dendritic branch points (e) and number of Syp I-positive (Syp I+) synaptic terminals (f) on the neuronal dendritic arbor within the region of interest (circular ROI, 50 µm radius from the center of the soma).

proteins like vesicular glutamate transporter 2 (VGluT2) and vesicular inhibitory amino acid transporter also known as the vesicular GABA transporter (VIAAT or VGAT, respectively) which are present specifically at the glutamatergic and GABAergic presynaptic terminals respectively. Finally, we determined the mRNA expression level of TrkB. The standard curves prepared for each gene analyzed are shown in Fig. 5. Table 3 details the numerical values for the slope and intercepts for the curves. The experiments indicated that added BDNF increased the expression levels of synaptophysin I, synapsin I and TrkB but decreased the mRNA levels of VGluT2 and VIAAT (Fig. 6), a result consistent with the previous data from collicular cultures (Henneberger et al., 2005). In all cases, the direction of the effects obtained with BDNF added to *bdnf-/-* cultures counteracted the changes resulting from chronic BDNF deficiency (compare data from *bdnf+/+* and *bdnf-/-*). Together, the results that added BDNF alters the number of synaptic terminals and dendrite morphology along with altered mRNA expression of synaptic proteins, support the notion that BDNF is strongly involved in the regulation of synapse density. However, these effects were produced by general presence of BDNF, which are expected to differ from those produced by a local concentration source of BDNF. The effects of the local action of spatially restricted BDNF were examined next.

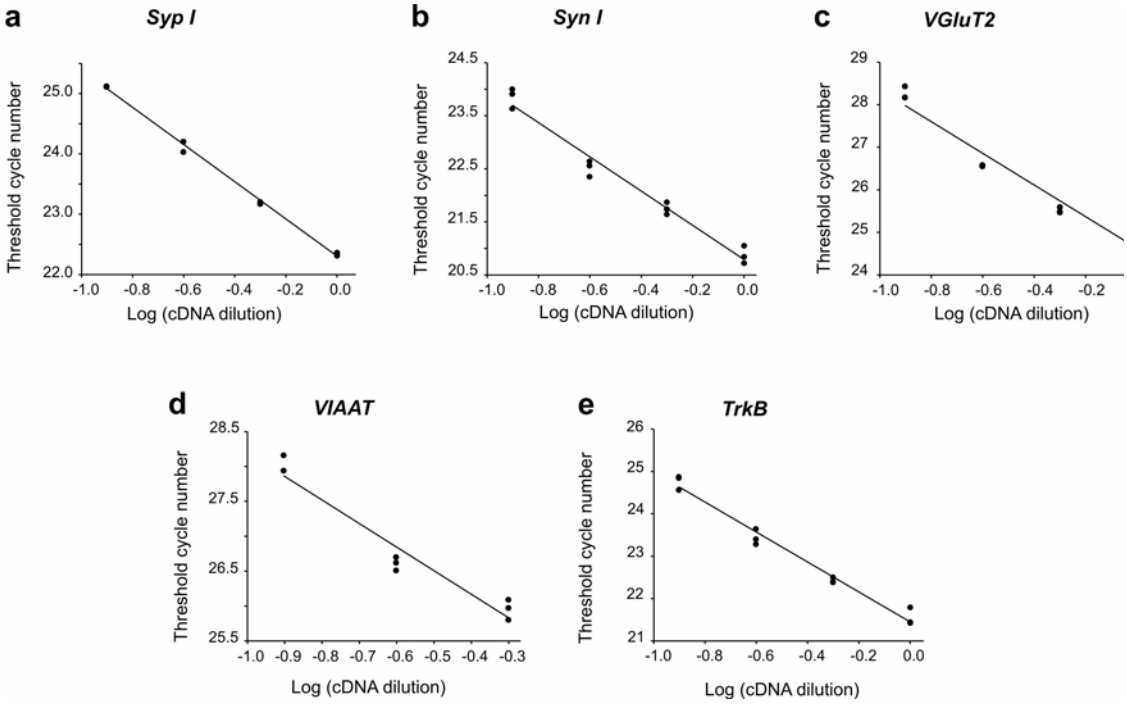

Figure 5. Standard curves for the analysis of mRNA expression levels of active zone proteins and TrkB. The mRNA expression profile of synaptophysin I (*Syp I*), synapsin I (*Syn I*), vesicular

glutamate transporter 2 (*VGluT2*), vesicular inhibitory amino acid transporter (*VIAAT*) and receptor *TrkB* was analyzed using quantitative real time RT-PCR. The standard curves were plotted as correlations between threshold cycle number (*y-axis*) and logarithm of cDNA dilution (a relative and accurate value, *x-axis*). r^2 is the regression coefficient. The numerical values for the slope and intercept are listed in the table below.

	Syp I	*Syn I*	*VGluT2*	*VIAAT*	*TrkB*	*GAPDH*
Slope	-3.081	-3.224	-3.713	-3.381	-3.53	-3.32
Intercept	22.304	20.792	24.629	24.816	21.449	25.32
r^2	0.99	0.97	0.95	0.93	0.98	0.99

Table 3. Numerical values of slope and intercept. The values were obtained from linear correlation diagrams used to plot a standard curve for each gene analyzed. Glyceraldehyde-6-phosphate dehydrogenase (GAPDH) was the control house keeping gene whose mRNA levels were used for normalization.

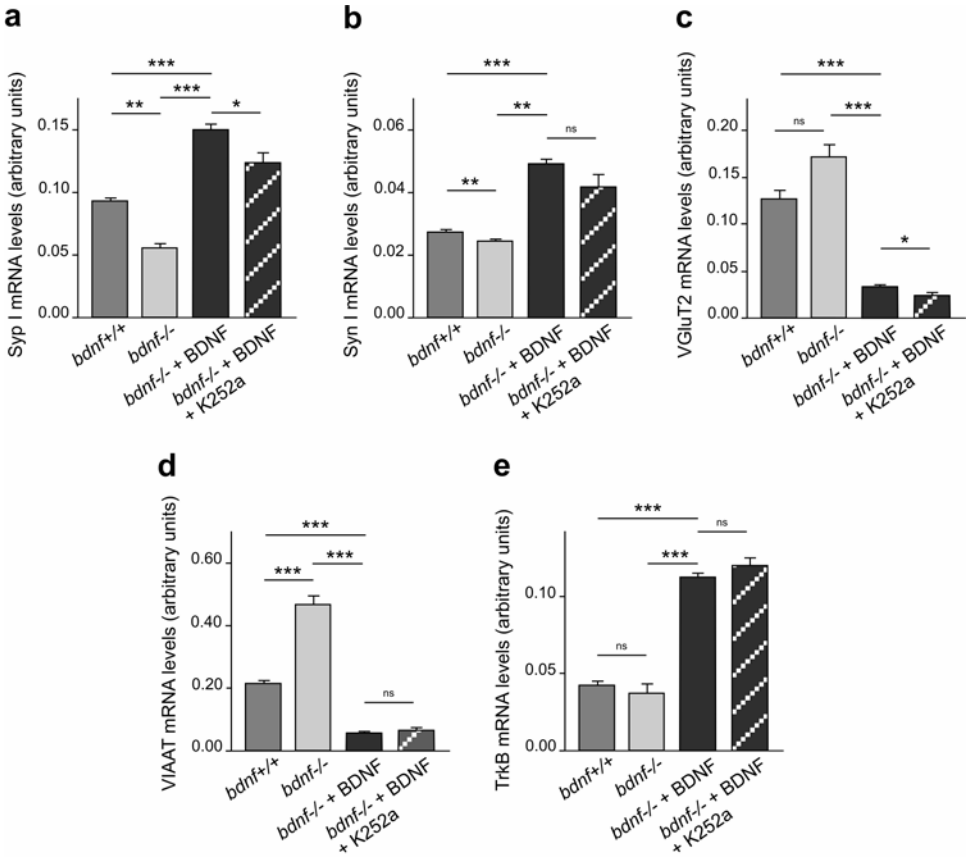

Figure 6. Exogenous addition of BDNF alters mRNA expression levels of the active zone proteins and receptor TrkB. Dissociated cultures from E18 *bdnf-/-* mouse hippocampus were made at an

intended cell density of 60, 000 cells/cm^2. On DIV 5, the neurons were treated with either a control medium or medium supplemented with BDNF (100 ng/ml) for 16 h. Cells were then harvested for RNA isolation. Quantitative real-time PCR was performed using SYBR-Green assay for transcript levels of **(a)** synaptophysin I (Syp I), **(b)** synapsin I (Syn I), **(c)** vesicular glutamate transporter 2 (VGluT2), **(d)** vesicular inhibitory amino acid transporter (VIAAT) and **(e)** receptor TrkB. mRNA expression levels were normalized against those for a house keeping gene, glyceraldehyde-6-phosphate dehydrogenase (GAPDH). **(a-b)** *bdnf* -/- cultures showed a reduction in the mRNA levels of Syp I and Syn I when compared to *bdnf* +/+ neurons consistent with the lower number of synaptic terminals present in -/- cultures. **(c-d)** VIAAT mRNA level increased significantly while VGluT2 showed an insignificant increase in -/- cultures. **(e)** TrkB mRNA levels in *bdnf-/-* cultures were indistinguishable from those in *bdnf* +/+ cultures. Addition of BDNF increased the transcript levels of Syp I, Syn I and TrkB, while VIAAT and VGluT2 levels were reduced significantly. Unspecific blocker of Trk tyrosine kinase receptors could not oppose the effects of added BDNF except for a slight but significant change in case of Syp I and VGluT2 mRNA. Significance levels are indicated as * $p<0.05$, and *** $p<0.001$, ns not significant.

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3. LOCAL ACTION OF TRANSFECTED BDNF

3.1. BDNF-expression drastically alters the dendrite morphology of transfected hippocampal neurons

To explore the consequences of local concentration of BDNF on dendrite morphology and synapse development, BDNF transfection in single hippocampal neurons from *bdnf-/-* mice, was performed. This investigation was started by studying the effects on dendrite morphology. Dissociated hippocampal neurons from *bdnf-/-* mice were transfected on DIV 5, with a BDNF::EGFP construct. The control neurons were transfected with the EGFP construct. After an expression time of 16 h, the cultures were fixed and immunostained for EGFP and MAP2. It was found that in some of the transfected neurons the EGFP signal emerging from transfected BDNF (t-BDNF) exhibited a diffuse distribution throughout the soma, dendrites and axons (Fig. 7a-b). However, in support of the studies by Haubensak et al., 1999 and Adachi et al., 2005, another population of BDNF::EGFP-transfected neurons, most neurons displayed clusters of BDNF::EGFP in the dendrites, in addition to diffuse labeling (Fig. 7c). A clustered distribution of BDNF suggests that BDNF is sorted into the regulated secretory pathway.

Evaluation of dendrite morphology showed that the BDNF::EGFP-transfected neurons differed from EGFP-transfected neurons. The former possessed a larger number of primary dendrites and branch points, but fewer dendritic trees with a length >50 µm (Fig. 7, 8).

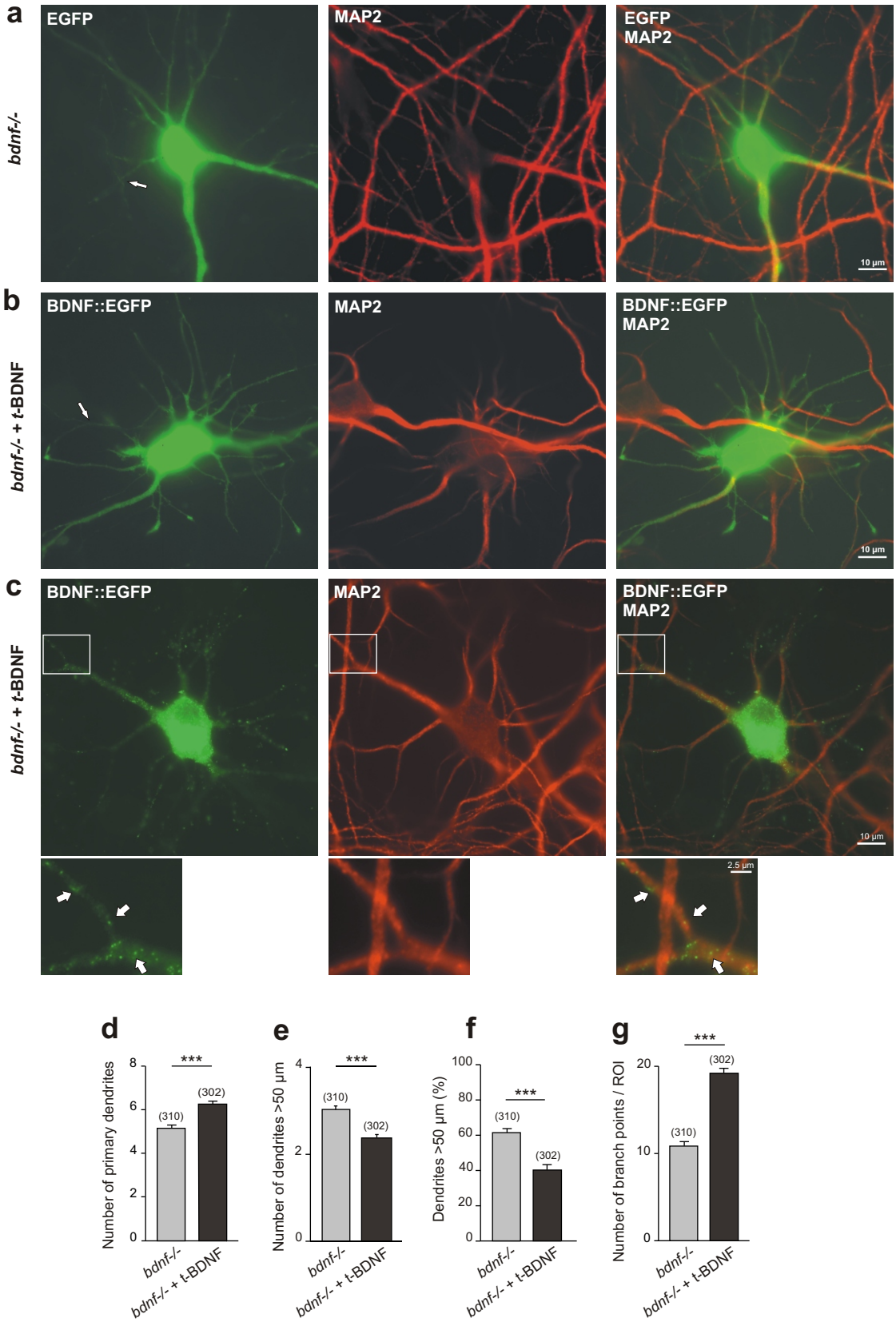


Figure 7. BDNF expression drastically changes neuronal morphology. (a-c) Immunofluorescence images of EGFP-transfected (a) or BDNF::EGFP transfected (t-BDNF; b-c). *bdnf*-/- neurons in culture were transfected on DIV 5 and after an expression time of 16 h,

immunocytochemistry was performed with antibodies against EGFP and MAP2. All EGFP positive neurites except one (small arrow, indicating possibly an axon) were MAP positive. BDNF::EGFP transfected neurons showed both diffuse (b) and punctate (c) staining for EGFP suggesting a difference in expression levels where strong EGFP fluorescence masking the granular localization of BDNF. Vesicular puncta of EGFP tagged-BDNF were apparently visible in the magnified view (c). Large arrows indicate the punctate distribution of BDNF::EGFP. Second image shows MAP2 staining of the neuron in first image. Third image is the superimposition of first two corresponding images. **(d-g)** Quantification of results obtained after BDNF::EGFP transfection. Transfected BDNF (t-BDNF) stimulated the formation of new primary dendrites (d), inhibited dendrite elongation as shown by the number and fraction of dendrites longer than 50 µm (e and f, respectively) and enhanced dendritic branching (g). Numbers in parentheses denote the number of neurons studied. Significance levels are indicated as * $p<0.05$, and *** $p<0.001$. ns not significant.

The number of primary dendrites in control neurons was found to be 5.1 ± 0.1 (n=310) but 6.2 ± 0.1 (n=302, $p<0.001$) in BDNF::EGFP-transfected neurons (Fig. 7d). The number of branch points also increased from 10.8 ± 0.2 to 19.2 ± 0.5 ($p<0.001$, Fig. 5g). As observed in the case of exogenous BDNF application, BDNF::EGFP transfection also reduced the fraction of dendritic trees >50 µm from 69.6 ± 7.8 % (control) to 40.2 ± 1.3% (transfected, Fig. 7f). The absolute number of the dendritic trees >50 µm was found to be 3.0 ± 0.06 in control and 2.4 ± 0.08 ($p<0.001$) in BDNF::EGFP-transfected neurons (Fig. 7e). The morphological changes after BDNF::EGFP expression were most pronounced within 50 µm distance from the soma, therefore not only the changes in dendrite geometry but also the number of synapses in contact with the labeled dendrites were analyzed within this region of interest, ROI (Fig. 8).

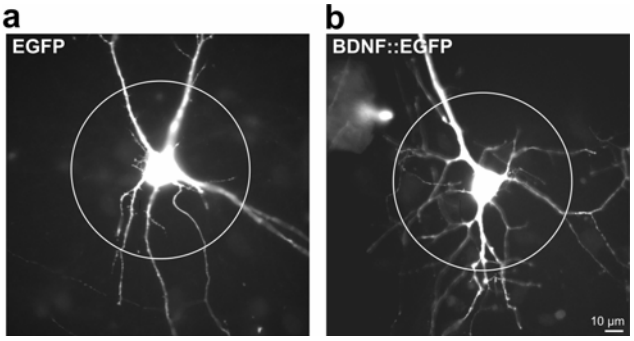


Figure 8. BDNF::EGFP-induced changes in dendrite morphology at lower magnification (40x objective). Morphological changes after BDNF::EGFP expression were most pronounced within 50 µm distance from the soma. (a) an EGFP-transfected and (b) a BDNF::EGFP -transfected *bdnf-/-* neuron on DIV 6 in culture. A circular viewfield (ROI) of diameter 100 µm is marked to illustrate the enhancement of dendritic branching within this region.

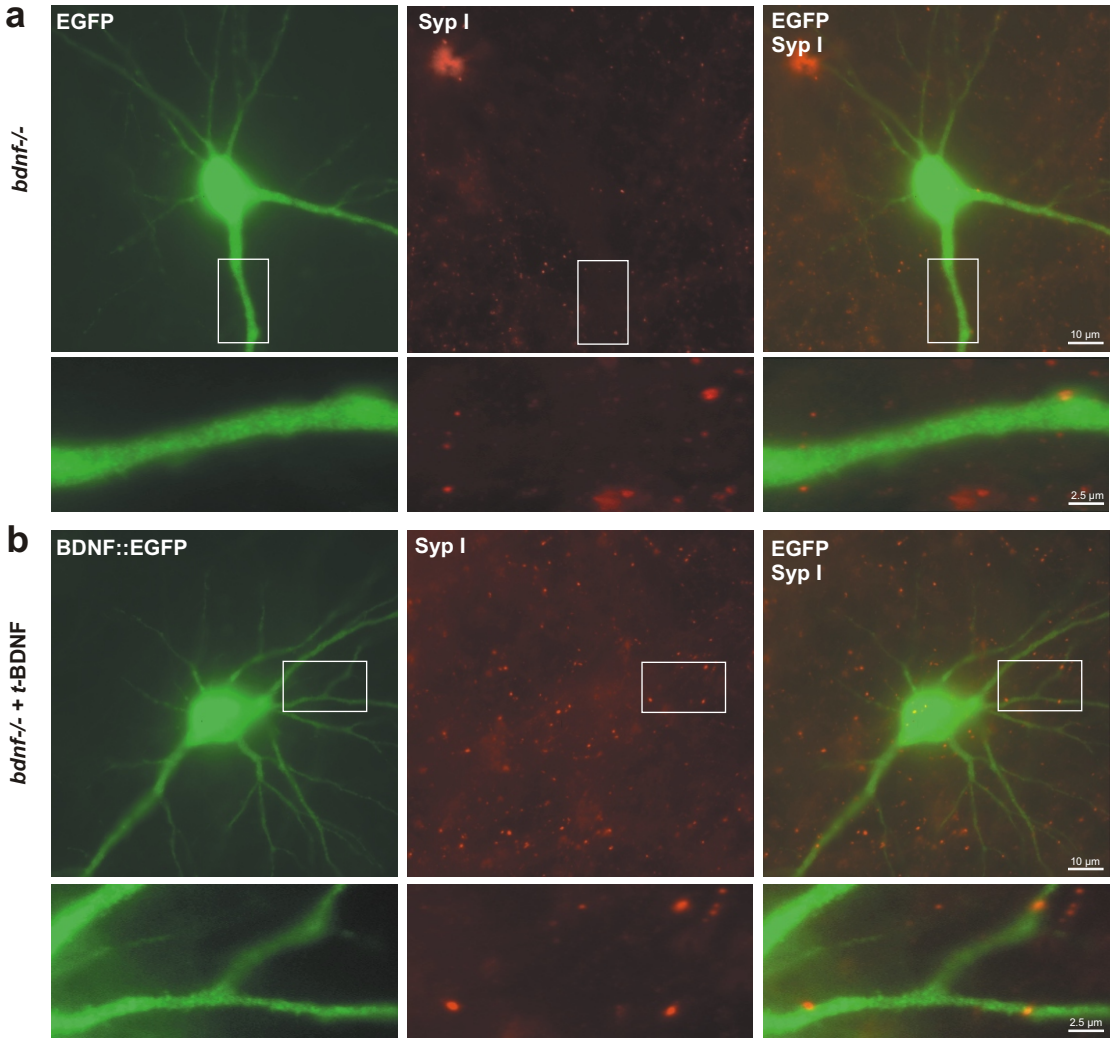
These results are consistent with the idea that BDNF expression can stimulate dendrite initiation but reduce dendrite elongation. Whether, this remarkable change in dendrite growth is coupled to a change in synapse formation was the issue that was addressed next.

3.2. BDNF-expression increases the number of synaptophysin I -positive terminals

Synapse formation was evaluated by quantifying the number of synaptic terminals present on *bdnf-/-* neurons expressing BDNF::EGFP as opposed to EGFP alone. To visualize the synaptic terminals, immunocytochemical staining was performed with an antibody against synaptophysin I (Syp I), a marker for presynaptic terminals (Fig. 9a-b). The Syp I-positive (Syp I+) puncta in contact with EGFP-labeled dendrites and somata were counted within the circular ROI (Fig. 9c). Quantification of the results yielded that the number of Syp I+ terminals was significantly higher in BDNF::EGFP-expressing neurons (20.4 ± 0.5, n=285 versus 10.5 ± 0.3, n=315 in control, $p<0.001$). Hence, these experiments suggested that neurons able to release BDNF can attract or stabilize many more synaptic terminals than neurons lacking BDNF.

3.3. BDNF-expressing neurons receive a larger number of glutamatergic terminals, but a smaller number of GABAergic terminals

The next set of experiments was aimed at answering the question that whether or not this increase in the number Syp I+ terminals was synapse-specific. Theoretically, the change could be due to either or both kinds of synapses. A combined immunostaining for Syp I and vesicular glutamate transporters-1 and -2 (VGluT1/2) was performed, and it was found that the increase induced by BDNF transfection was even more pronounced when counting glutamatergic terminals, instead of all Syp I+ terminals in contact with an EGFP-labeled neuron (Fig. 10a, b). Next, the specific effect of postsynaptic BDNF expression on inhibitory synaptic terminals was examined. The latter were visualized by immunostaining for vesicular inhibitory amino acid transporter (VIAAT) and Syp I (Fig. 11a). These experiments showed that postsynaptic BDNF expression resulted in a smaller number of GABAergic terminals received by the neurons (Fig. 11b).



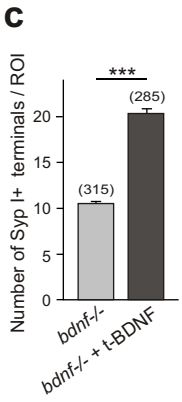


Figure 9. BDNF expressing neurons have a higher number of synaptic terminals than that in BDNF-deficient neurons. (a-b) Immunofluorescence image of an EGFP- (a) or BDNF::EGFP- expressing neuron (t-BDNF, b) from E18 *bdnf-/-* hippocampal cultures, fixed and immunostained for EGFP and synaptophysin I (Syp I) on DIV 6 after 16 h of gene expression following transfection with EGFP or BDNF::EGFP. Below is the magnified image of the boxed area in the corresponding image. Original black and white digital images (1035 x 1035 pixels) were pseudocolored as green (EGFP or BDNF::EGFP) and red (Syp I). The third image is the overlay of the first two showing the Syp I positive puncta contacting the dendritic tree of the neuron within the region of interest (ROI). **(c)** Increase in the number of Syp I positive (Syp I+) synaptic terminals in BDNF-transfected (t-BDNF) neurons after 16 h of gene expression when compared to the control *bdnf-/-* neurons which expressed EGFP. Numbers in parenthesis denote the number of neurons studied for the respective treatment group. Significance levels are indicated as *** $p<0.001$.

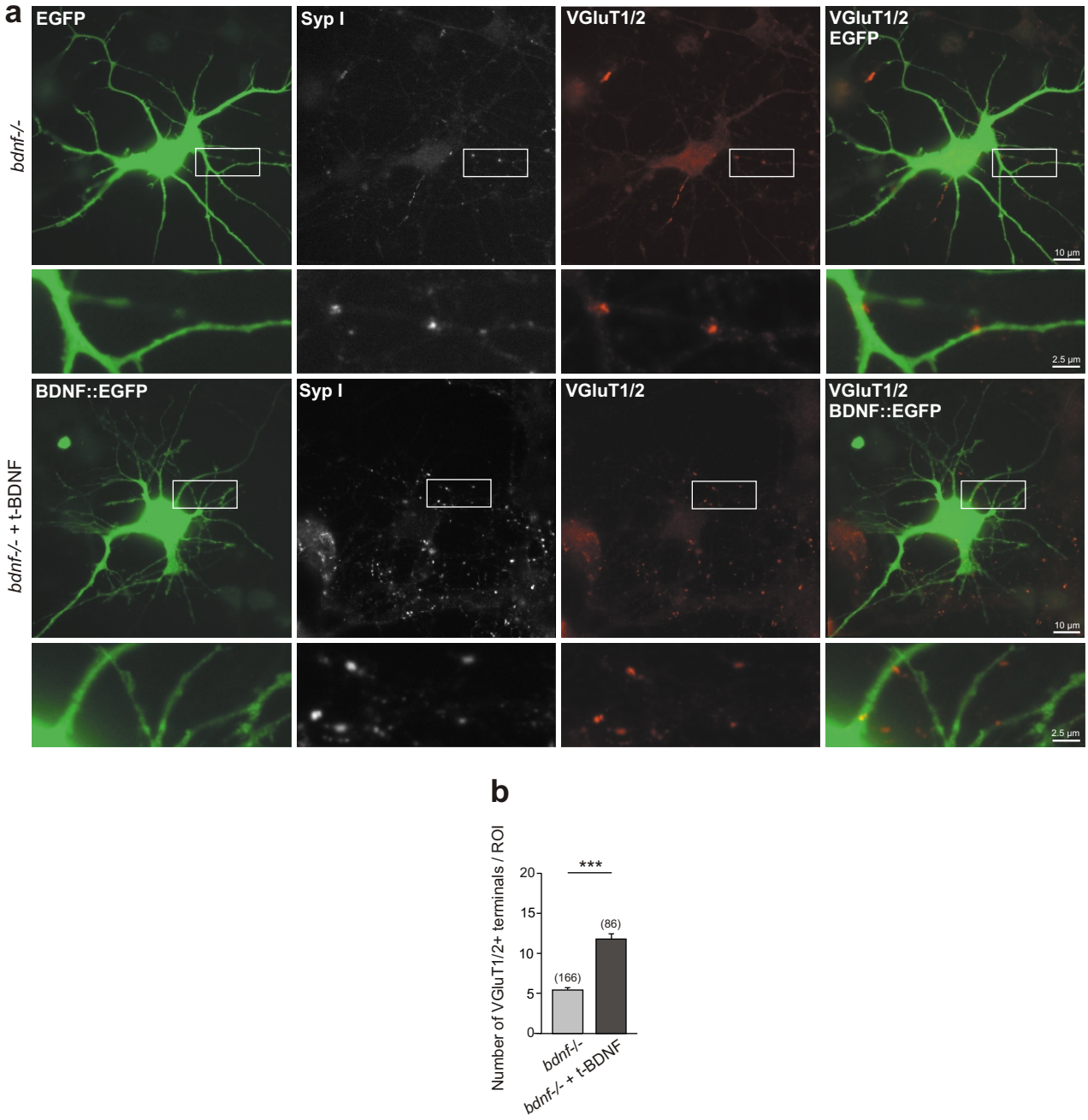


Figure 10. BDNF expressing neurons receive a larger number of glutamatergic synaptic terminals.

(a) Immunofluorescence images of *bdnf-/-* neurons expressing either EGFP (above) or BDNF::EGFP (t-BDNF, below). EGFP immunoreactivity is shown in green, synaptophysin I (Syp I) in white and vesicular glutamate transporter 1 and 2 (VGluT1/2) in red. Below are the magnified images of the boxed viewfields. VGluT1/2 immunoreactive puncta which were also positive for Syp I and were present on the dendritic tree within the region of interest (ROI, 50 µm from center of the soma) were counted as excitatory glutamatergic synaptic contacts. **(b)** Quantification of the data revealed an increase in the number of excitatory synaptic contacts on the BDNF expressing neurons (t-BDNF) when compared to the non-expressing neurons. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as *** $p<0.001$.

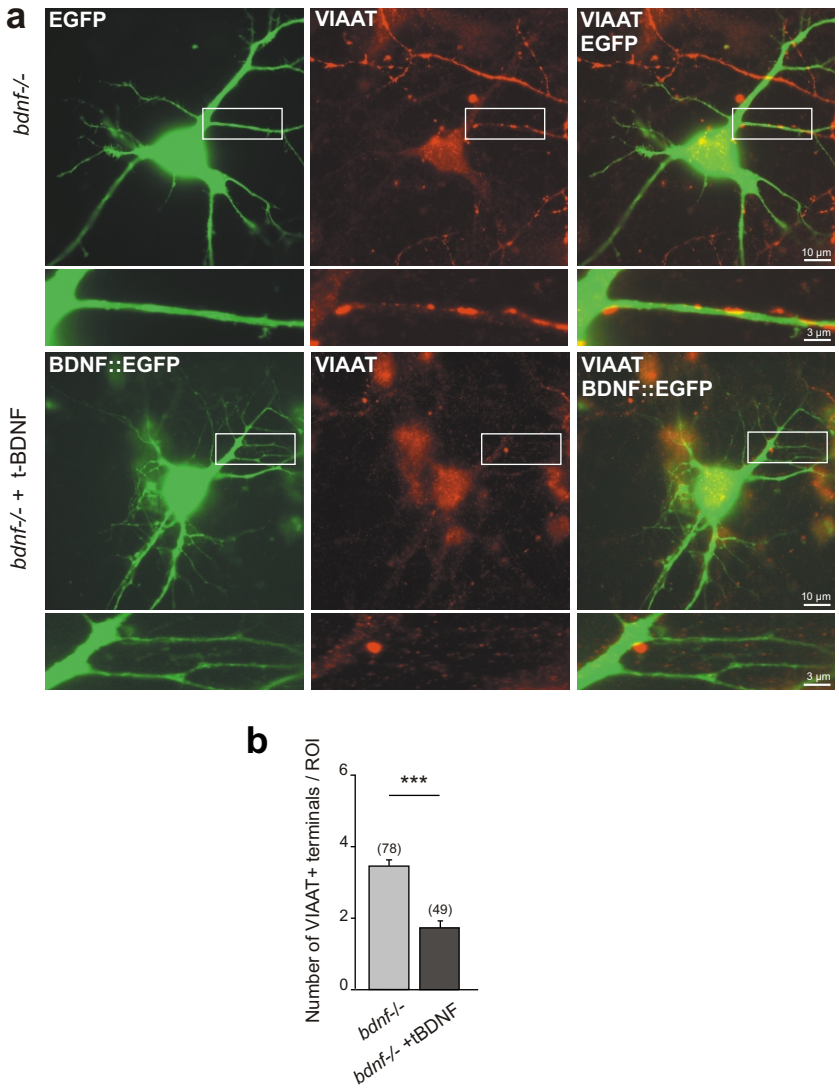


Figure 11. BDNF expressing neurons receive a smaller number of GABAergic synapses. **(a)** Immunofluorescence images of *bdnf-/-* neurons expressing either EGFP (upper panel) or BDNF::EGFP (t-BDNF, lower panel). EGFP immunoreactivity is shown in green and vesicular inhibitory amino acid transporter (VIAAT) in red. Below are the magnified images of the boxed viewfields. VIAAT immunoreactive (VIAAT+) terminals which were also positive for Syp I and were present on the dendritic tree within the region of interest (ROI, 50 µm radius from center of the soma) were counted as inhibitory GABAergic synaptic contacts. **(b)** Quantification of the data revealed a decrease in the number of GABAergic synaptic contacts on the BDNF expressing neurons (t-BDNF) when compared to the non-expressing neurons. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as*** $p<0.001$.

3.4. BDNF shifts the excitatory-to-inhibitory (E/I) ratio of synaptic terminal number

A persistent upregulation in the number of excitatory glutamatergic synaptic terminals and a downregulation in the number of GABAergic synaptic terminals, is expected to boost neuronal excitation and to rise the intracellular calcium concentration with possible excitotoxic consequences. It was therefore asked whether neurons could maintain an increase of the E/I ratio for a longer period of time (72 h). This evaluation only includes those neurons for which both the E and I numbers were counted in one and the same neuron. Indeed, the experiments revealed significantly higher E/I values in BDNF-expressing neurons (Fig. 12g). The respective values were 3.0 ± 0.3 in BDNF-transfected neurons (t-BDNF, n=20) as opposed to 1.6 ± 0.3 in BDNF-deficient neurons (*bdnf-/-*, n=25). The ratio of VGluT1/2 positive terminals to the total number of synaptic terminals (sum of VGluT1/2-positive and VIAAT-positive terminals) was 0.74 ± 0.1 in BDNF-transfected neurons as opposed to 0.54± 0.1 in BDNF-deficient neurons. This differential action of local postsynaptic BDNF on glutamatergic (E) versus GABAergic (I) synaptic terminals in contact with the same cell is a major new result which, however, raises additional questions about the signaling pathways mediating such reciprocal effects.

3.5. Comparison of synaptogenesis in wild type and BDNF-deficient neurons

To compare the effect of local, postsynaptic BDNF with the effects of ubiquitous BDNF in normal preparations, the respective synapse numbers were determined in wild-type (*bdnf+/+*), BDNF-deficient (*bdnf-/-*), BDNF-supplemented *bdnf-/-* neurons and in BDNF-transfected *bdnf-/-* neurons. The results are summarized in Fig. 13. It can be seen that the synapse numbers observed in wild-type neurons could only be recoverd with BDNF-transfection, not with exogenous BDNF (Fig. 13 a, b). Interestingly, such recovery was not observed with regard to the number of inhibitory synaptic terminals, at least when BDNF-expression was limited to only 16 h (Fig. 13c). Summary of the dendritic parameters is also given in Table 4.

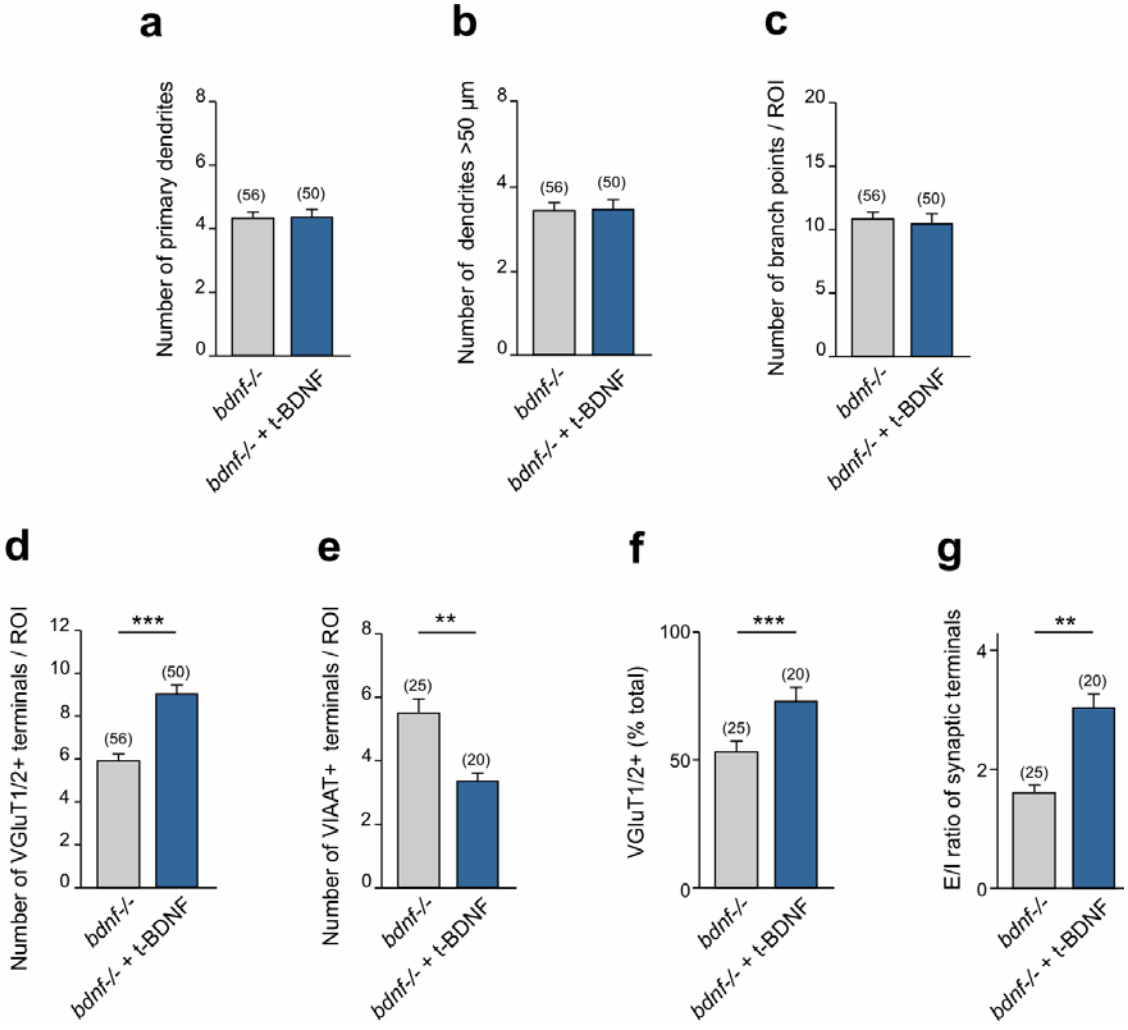


Figure 12. Effects of transfected BDNF on dendrite morphology after long term expression. (a-c) Quantification of the data obtained from analyses of neurons expressing BDNF::EGFP or EGFP for an expression time of 72 h. BDNF expressing neurons (t-BDNF) did not differ in their dendritic structure from the control neurons after long term expression. **(d)** Increase in the number of VGluT1/2 positive (VGluT1/2+) synaptic terminals in BDNF-transfected neurons. **(e)** Decrease in the number of VIAAT positive (VIAAT+) synaptic terminals in BDNF-transfected neurons. **(f)** Increase in the fraction of VGluT1/2+ synaptic terminals. **(g)** E/I (excitatory/inhibitory) ratio of VGluT1/2+ to VIAAT+ synaptic terminals increased in BDNF-transfected neurons. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

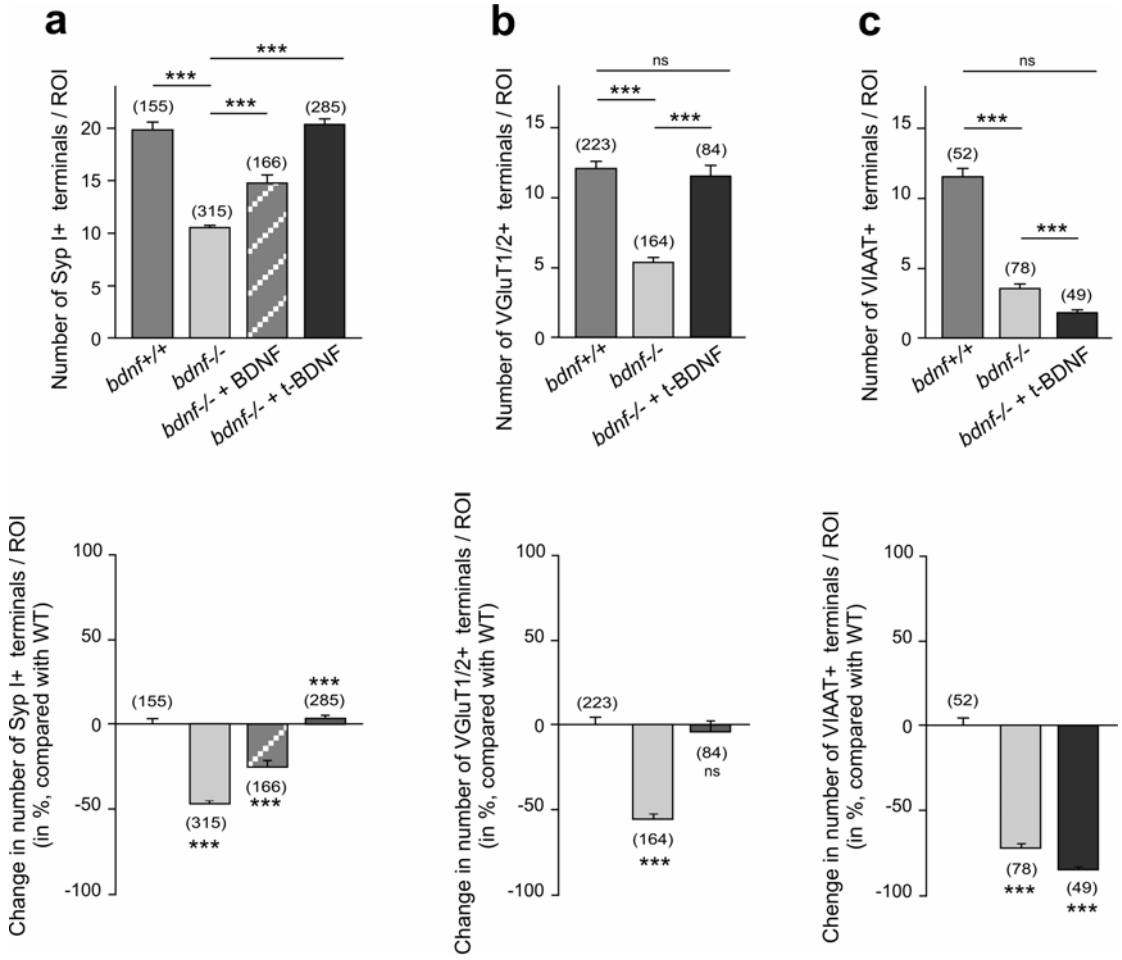


Figure 13. Comparison of synaptogenesis between *bdnf*+/+, *bdnf*-/- and BDNF-transfected (t-BDNF) *bdnf*-/- neurons. (a) *bdnf*-/- neurons exhibited a decline in total number of synaptophysin I-positive (Syp I+) synaptic terminals which was partially increased in neurons treated with exogenous BDNF (100 ng/ml). The number of Syp I+ synaptic terminals reached to the wild type level in *bdnf*-/- neurons transfected with BDNF::EGFP. **(b)** A similar pattern was observed for the glutamatergic synaptic terminals (VGluT1/2+ terminals) in the *bdnf*+/+, *bdnf*-/- and BDNF-transfected *bdnf*-/- neurons, where reduction in glutamatergic synapse formation in *bdnf*-/- neurons was made up to the wild type level in transfected neurons. **(c)** Decrease in the number of VIAAT positive (VIAAT+) GABAergic terminals in *bdnf*-/- neurons, which further decreased after BDNF-expression. Percent change in the number of Syp I+, VGluT1/2+ and VIAAT+ terminals from that in the *bdnf*+/+ neurons is shown in the corresponding graphs in the lower panel. Numbers in parentheses denote the number of neurons studied for the respective analyzed group. Significance levels are indicated as *** $p<0.001$.

Parameters studied	***bdnf*+/+** (Mean ± SEM, n= 155)	***bdnf*-/-** (Mean ± SEM, n=315)	***bdnf*-/- + BDNF** (Mean ± SEM, n= 166	***bdnf*-/- + t-BDNF** (Mean ± SEM, n=285)
Number of primary dendrites	4.2 ± 0.1	4.6 ± 0.1	5.8 ± 0.2 ***	6.1 ± 0.1 ***
Number of dendrites > 50 µm	3.1 ± 0.1	2.9 ± 0.1	2.1± 0.1	2.4 ± 0.1
Fraction of dendrites > 50 µm	78.1 ± 1.9%	65.3 ± 1.2% ***	40.5 ± 2.1% ***	40.9 ± 1.4% ***
Number of dendritic branch points per ROI	10.3 ± 0.3	10.0± 0.2	12.5± 0.4 *	19.0 ± 0.6 ***
Number of Syp I+ synaptic terminals per ROI	19.8 ± 0.7	10.5 ± 0.3 ***	14.8 ± 0.7 ***	20.4 ± 0.5 ***

Table 4. Comparison of dendrite morphology and synaptogenesis among *bdnf*+/+, *bdnf*-/- and BDNF-transfected (t-BDNF) *bdnf*-/- neurons. Various parameters of dendrite morphology and the number of synaptic terminals for *bdnf*-/- neurons, *bdnf*-/- neurons treated with exogenous BDNF (*bdnf*-/- + BDNF) and *bdnf*-/- neurons transfected with BDNF::EGFP (*bdnf*-/- + t-BDNF) were compared with those of *bdnf*+/+ neurons. Each value represents the value for an average individual neuron from the respective experimental group.

4. RELATIONSHIP BETWEEN DENDRITE MORPHOLOGY AND SYNAPTIC INPUT

4.1. Effects of neurotrophin receptor block on dendrite morphology and synaptic terminal number

From the transfection experiments, it was concluded that BDNF::EGFP expression drastically alters both dendrite growth and synapse formation in primary hippocampal neurons from *bdnf*-/- mice. To clarify whether changes in dendrite morphology and synapse numbers are interrelated, it was first sought to identify the respective neurotrophin receptors. TrkB was blocked either by a specific blocking antibody (TrkB IgG1) or by K252a (200 nM), an unspecific blocker of the Trk tyrosine kinase. p75 function was blocked by another specific antibody (p75-Ab). It was found that block of TrkB with K252a (Fig. 14b) or TrkB IgG (Table 5b) had little effect on the outgrowth of additional primary dendrites, but it prevented the t-BDNF-induced suppression of dendrite elongation (Fig. 14d). In contrast, the function block of p75 had no effect on the t-BDNF-induced suppression of dendrite elongation (Fig. 14c), but it reduced t-BDNF-induced hyperbranching (Fig. 14e-f).

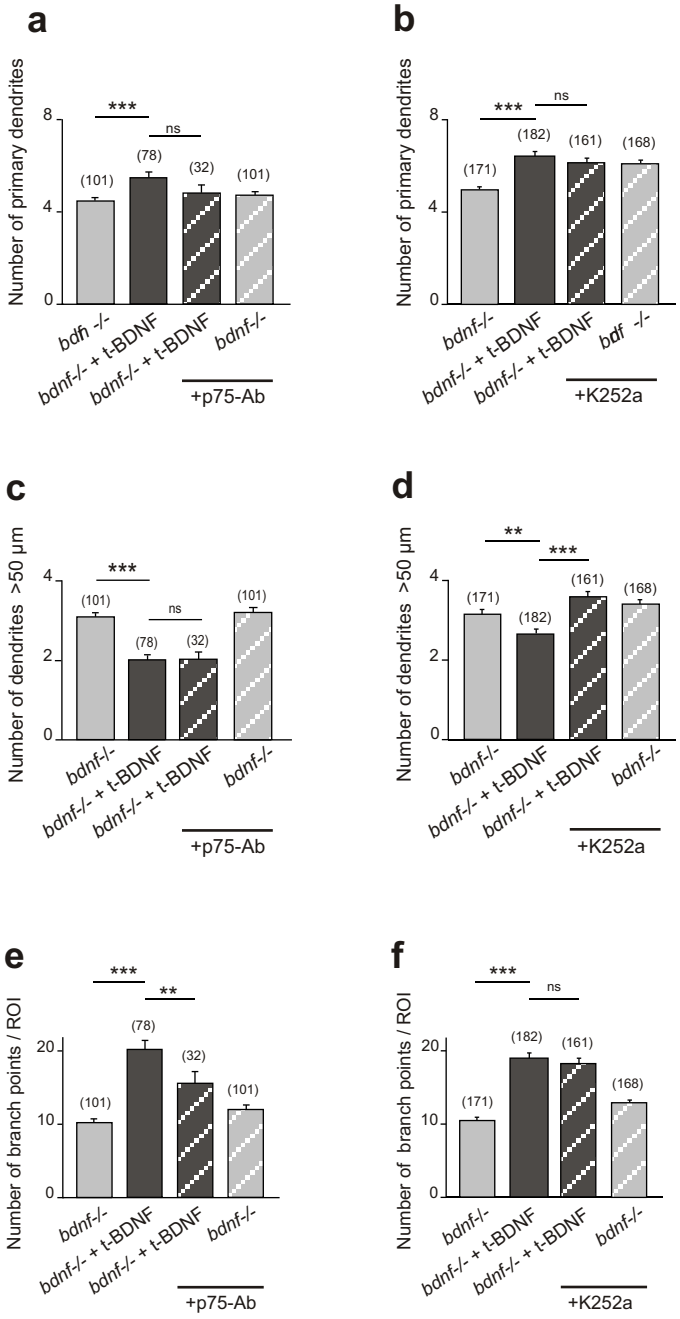


Figure 14. Receptor specific effects of local BDNF on dendrite elongation and branching. (a-b) Outgrowth of additional primary dendrites was not affected by block of p75 with blocking antibody (p75-Ab) (a) or block of Trk tyrosine kinase with K252a (b). Dendrite elongation as measured by the number of dendritic trees > 50 µm, in BDNF**::**EGFP-transfected (t-BDNF) neurons from *bdnf-/-* mice, was sensitive to K252a (d) but not to p75-Ab (c). **(e-f)** Dendrite branching on the other hand was sensitive to the block of p75 (f) but not to Trk receptor block (f). This indicates the involvement of TrkB signaling in the BDNF-mediated suppression of dendrite elongation and p75 signaling in enhancement of dendrite branching. p75-Ab was used at a dilution of 1:500 and K252a was used at a concentration of 200 nM. All blocking agents were applied to the culture medium for a treatment time of 16 h. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

a. Experiments on p75 block

Parameters studied	***bdnf-/-*** (Mean ± SEM, n= 101)	***bdnf-/-*** **+ t-BDNF** (Mean ± SEM, n=78)	***bdnf-/-*** **+ t-BDNF + p75-Ab** (Mean ± SEM, n= 32)	***bdnf-/-*** **+ p75-Ab** (Mean ± SEM, n=101)
Number of primary dendrites	4.5 ± 0.1	5.5 ± 0.2 ***	4.8 ± 0.4	4.8 ± 0.2
Number of dendrites > 50 µm	3.1 ± 0.1	2.0 ± 0.1 ***	2.0 ± 0.2	3.2 ± 0.1
Fraction of dendrites > 50 µm	70.6 ± 2.2%	39.1 ± 2.5% ***	47.9 ± 5.5% ***	70.5 ± 2.1%
Number of dendritic branch points per ROI	10.2 ± 0.5	20.2 ± 1.2 ***	15.4 ± 1.6 **	12.2 ± 0.6
Number of synaptic terminals per ROI	11.0 ± 0.5	23.3 ± 0.9 ***	23.7 ± 1.4	18.4 ± 0.7

b. Experiments on TrkB block with TrkB IgG

Parameters studied	***bdnf-/-*** (Mean ± SEM, n=43)	***bdnf-/-*** **+ t-BDNF** (Mean ± SEM, n=25)	***bdnf-/-*** **+ t-BDNF + TrkB IgG** (Mean ± SEM, n=27)	***bdnf-/-*** **+ TrkB IgG** (Mean ± SEM, n=30)
Number of primary dendrites	4.5 ± 0.2	6.2 ± 0.2 ***	5.2 ± 0.3 *	4.7 ± 0.3
Number of dendrites > 50 µm	3.1 ± 0.2	2.2 ± 0.2 **	2.5 ± 0.2	2.9 ± 0.2
Fraction of dendrites > 50 µm	72.0 ± 3.5%	36.1 ± 3.6% ***	54.1 ± 5.9% **	63.4 ± 4.3%
Number of dendritic branch points per ROI	10.2 ± 0.8	14.4 ± 1.5, ***	10.8 ± 0.9	10.4 ± 0.7
Number of synaptic terminals per ROI	11.5 ± 0.8	17.4 ± 1.2, ***	9.9 ± 1.0	11.2 ± 0.8

c. Experiments on Trk receptor block with K252a

Parameters studied	***bdnf-/-*** (Mean ± SEM, n=171)	***bdnf-/-*** **+ t-BDNF** (Mean ± SEM, n=182)	***bdnf-/-*** **+ t-BDNF + K252a** (Mean ± SEM, n=161)	***bdnf-/-*** **+ K252a** (Mean ± SEM, n=168)
Number of primary dendrites	4.9 ± 0.1	6.4 ± 0.2 ***	6.1 ± 0.2	6.0 ± 0.2
Number of dendrites > 50 µm	3.1 ± 0.1	2.6 ± 0.1 **	3.6 ± 0.1 ***	3.4 ± 0.1
Fraction of dendrites > 50 µm	64.4 ± 1.9%	42.3 ± 1.8% ***	61.3 ± 2.0% ***	59.4 ± 1.7%
Number of dendritic branch points per ROI	10.5 ± 0.4	19.1 ± 0.7 ***	18.3 ± 0.7 ***	13.0 ± 0.4
Number of synaptic terminals per ROI	10.2 ± 0.5	19.5 ± 0.6 ***	5.8 ± 0.3 ***	7.6 ± 0.3

Table 5. Summary of the effects of neurotrophin receptor block on BDNF::EGFP-induced changes in dendrite morphology and synaptic terminal number. *bdnf-/-* primary hippocampal

neurons were transfected with BDNF::EGFP and treated with p75-Ab (a), TrkB IgG (b) or K252a (c) in three separate sets of experiments. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * p<0.05, ** p<0.01 and *** p<0.001. Comparison was done among the *bdnf-/-* and BDNF::EGFP transfected neurons and the latter with the BDNF::EGFP transfected treated with respective blocking agent.

The consequences of the NT receptor block were also evaluated with regard to synapse number (Fig. 15) and it was found that the t-BDNF-induced upregulation was sensitive to TrkB but not p75 blockade. Similar effects were found in counts of VGluT1/2-positive terminals. Interestingly, the suppressive effect of t-BDNF on the number of VIAAT-positive terminals was also sensitive to block of TrkB (Fig. 16). These results indicate that BDNF differentially regulates synapse formation through TrkB signaling.

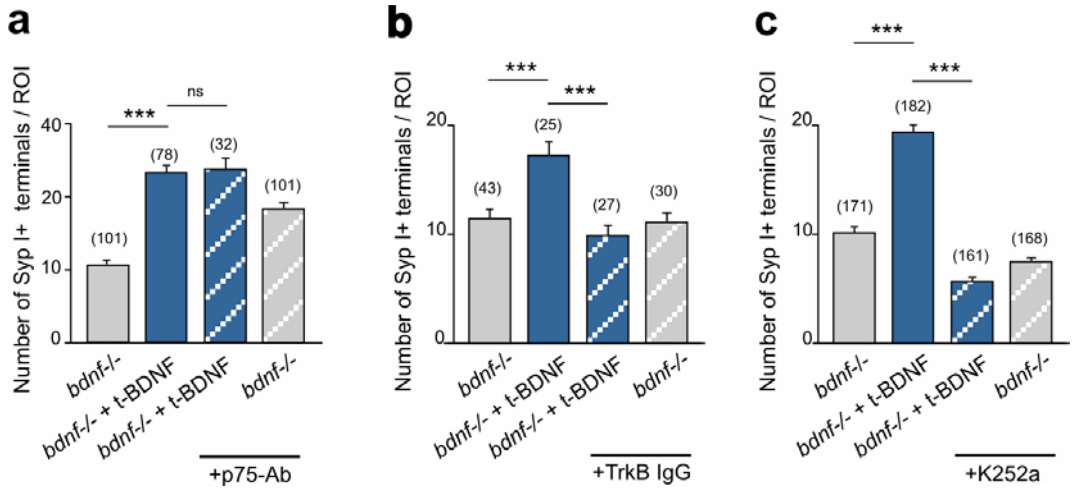


Figure 15. Receptor-specific effects of BDNF expression on synapse formation. Transfection of BDNF (t-BDNF) in *bdnf-/-* neurons increased the number of synaptic terminals as detected by synaptophysin I immunoreactive (Syp I+) terminals on the dendritic arbor within the ROI (ROI, 50 µm radius from the center of the soma). **(a-c)** The enhancement of synaptic terminal number after 16 h of BDNF expression was resistant to the block of p75 (a) but sensitive to the block of TrkB with TrkB IgG1 (b) and K252a (c). This suggests a specific role of TrkB signaling in mediating the effect of BDNF expression on synapse formation. Anti p75 antisera (p75-Ab) was used at a dilution of 1:500, TrkB IgG1 at concentration of 0.5 µg/ml and K252a at 200 nM. All blocking agents were applied to the culture medium for a treatment time of 16 h. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * p<0.05, ** p<0.01 and *** p<0.001.

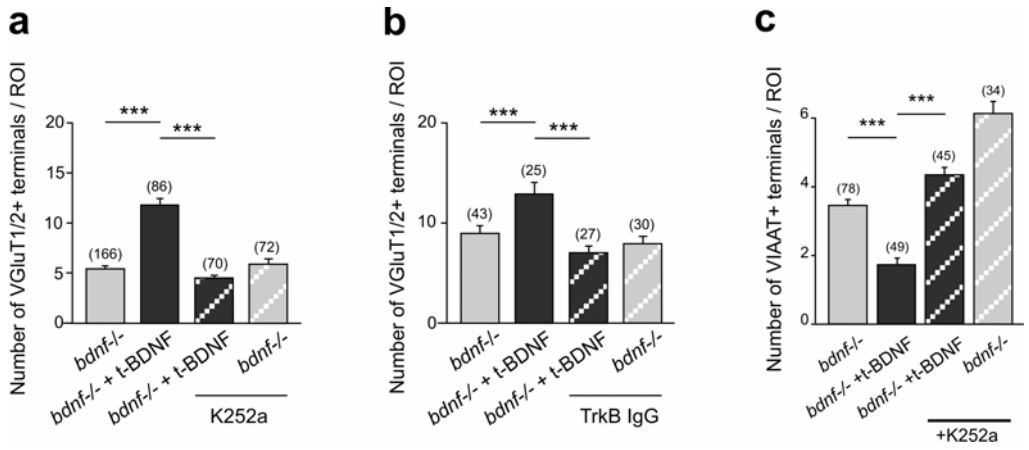


Figure 16. Receptor-specific differential effects of BDNF expression on glutamatergic and GABAergic synapse formation. (a-b) The enhancement in glutamatergic terminal number by t-BDNF was prevented in the presence of TrkB blockers, K252a (a) and TrkB blocking antibody, TrkB IgG (b). Similarly, the t-BDNF-induced decrease in the number of VIAAT positive terminals in t-BDNF neurons was blocked after treatment with K252a (c).

4.2. Effect of glutamate receptor block on dendrite morphology and synaptic terminal number

As there is evidence that secretion of BDNF is regulated by neuronal activity, it was sought to be determined if the BDNF-mediated responses in BDNF::EGFP-transfected *bdnf-/-* neurons were dependent on the function of glutamate receptors (GluRs). Therefore, the EGFP-transfected or BDNF::EGFP-transfected neurons were treated with a mixture of GluR antagonists, namely, DNQX (10 µM), MK801 (1 µM) and S-MCPG (200 µM). The evaluation showed that the block of GluR activity antagonized the effect of t-BDNF on dendrite growth (Fig. 17a-c). But, the number of VGluT1/2-positive terminals was further enhanced by the GluR block (Fig. 17d), consistent with the homeostatic concept that block of glutamatergic synaptic transmission increases synaptic glutamate release. The final result corresponded to about a sum of the individual effects of TrkB and GluR block.

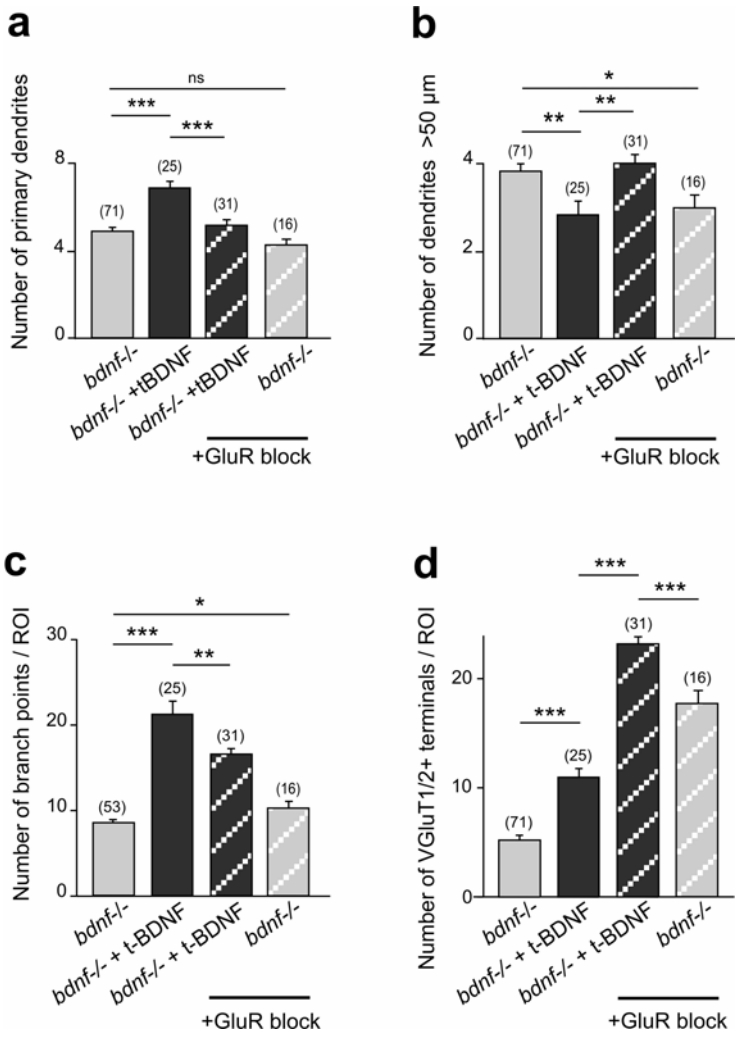


Figure 17. BDNF regulates dendrite morphology and synapse formation through separate GluR activity-dependent and -independent pathways. Effects of glutamate receptor (GluR) block on primary dendrite formation (a), dendrite branching (c), and glutamatergic terminal number (d). **(a-b)** In BDNF::EGFP-expressing neurons (t-BDNF), the effects of BDNF on enhanced primary dendrite formation and suppressed dendrite elongation were completely abolished suggesting that BDNF mediates primary dendrite formation and elongation through activity-dependent pathway. **(c)** Effects of GluR block on dendrite branching. BDNF-mediated increased dendrite branching was partially reduced but not completely opposed after blocking GluRs suggesting an additional activity-independent mechanism mediating dendritic branching. **(d)** Effects of GluR block on glutamatergic synapse formation. Block of GluRs increased the number of VGluT1/2 positive (VGluT1/2+) terminals in both BDNF::EGFP-transfected and control neurons. Block of glutamate receptors with DNQX (10 µM), MK801 (1 µM) and S-MCPG (200 µM) increased glutamatergic synapse numbers in both control and BDNF-expressing neurons, but in the latter this block had a more pronounced effect

indicating a role of BDNF in excitatory synapse formation. All blocking agents were applied to the culture medium for a treatment time of 16 h. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.3. Relationship between dendritic growth and glutamatergic synaptic input

The results described in 4.1 and 4.2 point to the interesting possibility that the t-BDNF-induced increment in the number of VGluT1/2-positive terminals is a cause of suppressed dendrite elongation. If true, one would expect a negative correlation between the number of dendritic trees exceeding the ROI and the number of VGluT1/2-positive terminals. This was, indeed, the case. Moreover, TrkB or GluR activity abolished this negative correlation (Fig. 18 b-c). Finally, in the presence of GluR blockers, t-BDNF failed to suppress dendrite elongation.

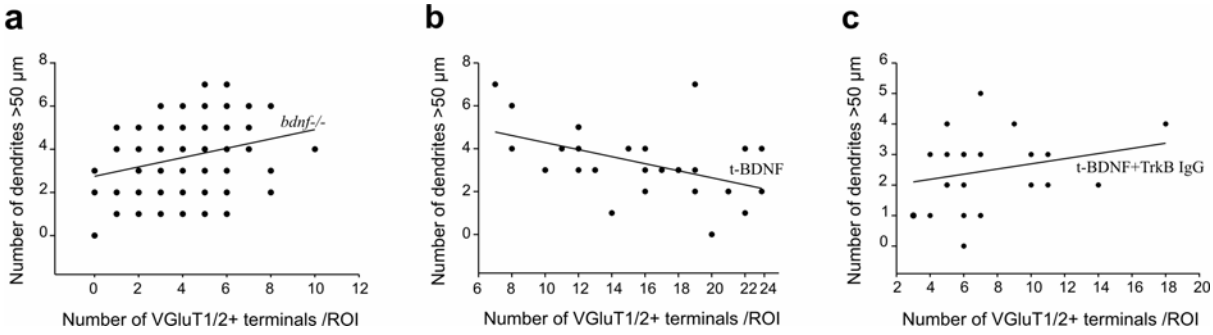


Figure 18. Scatter plots to show correlation between glutamatergic synaptic input number and dendrite morphology. (a) A positive correlation between the number of glutamatergic synaptic terminal number and number of dendritic trees >50 µm (a measure of dendrite elongation) in *bdnf-/-* neurons. Regression line for *bdnf-/-*, r=0.27, n=112, $p<0.01$. **(b)** A negative correlation between the number of VGluT1/2 positive (VGluT1/2+) terminals and number of dendritic trees exceeding 50 µm in BDNF::EGFP-transfected (t-BDNF) neurons. Regression line for t-BDNF, r=0.48, n=33, $p<0.01$. **(c)** Reversal of negative correlation shown in 'b' to a positive case, after treatment of BDNF::EGFP-transfected neurons with TrkB IgG1 for 16 h. Regression line for t-BDNF + TrkB IgG, r=0.28, n=30, $p>0.05$. TrkB blocking antibody, TrkB IgG was used at a concentration of 0.5 µg/ml.

5. ROLE OF PRG-1 IN SYNAPSE DEVELOPMENT

5.1. PRG-1 overexpression prevents LPA-induced loss of synapses

The starting point for this investigation was the observation that axotomized entorhinal cortical neurons displayed an upregulation of PRG-1 (Savaskan et al., unpublished results). A well-established component of a neuron's response to axotomy is synapse loss (Jones et al., 1997). Thus, the possibility exists that PRG-1 is required for the recovery or stabilization of synaptic inputs to axotomized cortical neurons that otherwise would lose the synaptic inputs. To investigate the possible role of PRG-1 in synapse development and stabilization, overexpression experiments were performed, by transfection of neurons with PRG1::EGFP construct. First hippocampal and later entorhinal cortical cultures were used for these experiments. As no differences were found between these preparations, the data were pooled in the graphs in Fig. 20, 22. The neurons were exposed to LPA, as it was known that this compound produces axonal retraction (Bräuer et al., 2003). It was asked whether or not the overexpression of PRG-1 would protect hippocampal/entorhinal cortical neurons against synapse loss after LPA challenge. A concentration range of LPA (0.1 µM-100 µM) functioned in a dose-dependent manner in reducing the number of synaptic contacts within the circular ROI (diameter 100 µm with soma at the center). The maximally effective extracellular concentration for added LPA was found to be 10 µM (Fig. 19c). Therefore, all subsequent experiments were performed using LPA at this concentration. There was no effect of LPA on neuronal survival, and the densities of neurons in cultures of the same age were comparable among the test and the control groups (Fig. 19d).

The evaluation showed that LPA and overexpression of PRG-1 had little effect on dendrite shape, apart from some degree of enhanced branching that was effectively abolished by t-PRG-1 (Fig. 20d). In contrast, LPA produced a marked reduction in the number of Syp I-positive terminals, and this loss was prevented by PRG-1 (Fig. 20e).

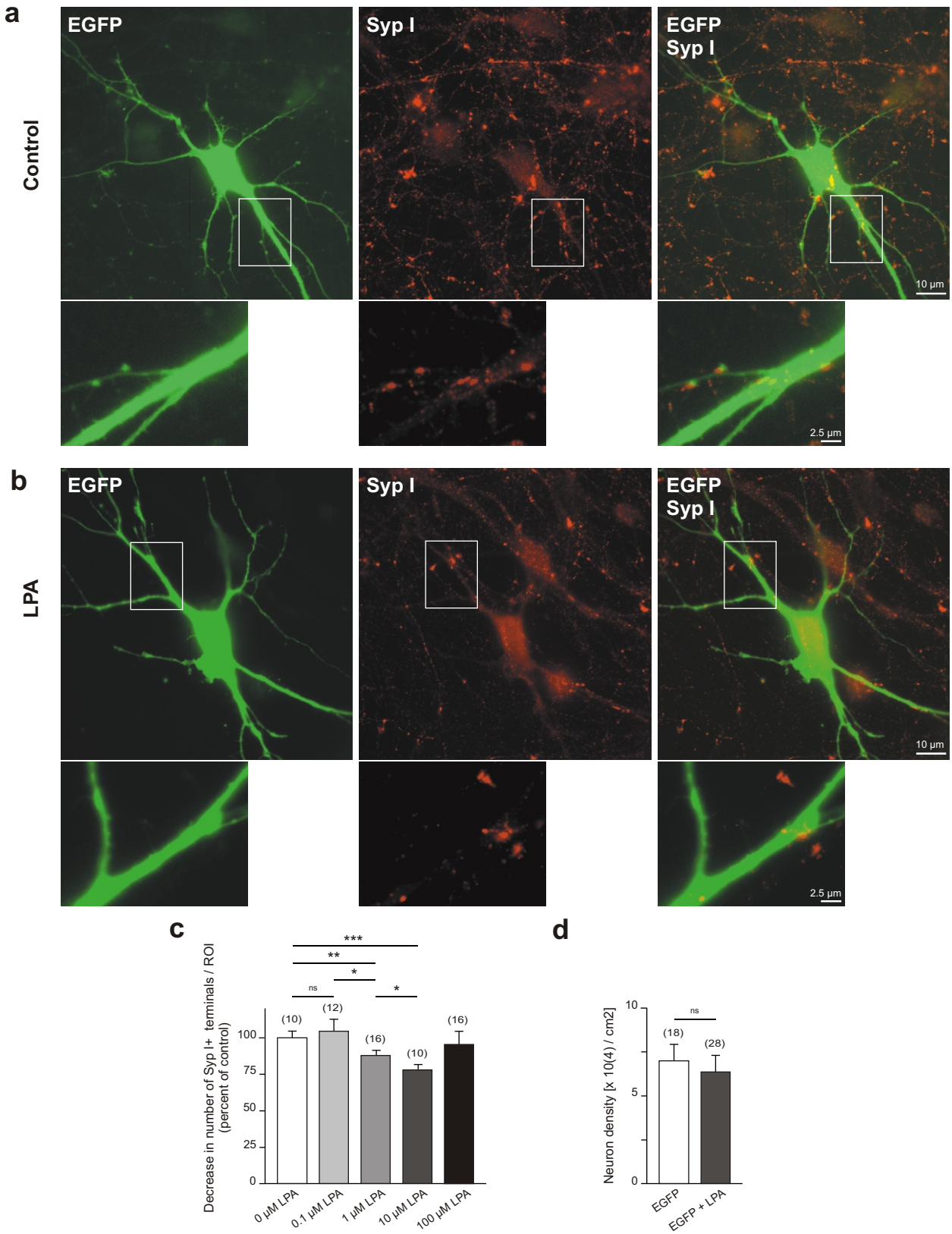


Figure 19. LPA application reduces the number of Syp I-positive terminals. (a-b) Immunofluorescence images of EGFP-tranfected neurons without (a) or with treatment with LPA (10 µM) (b). On DIV 5, primary hippocampal neurons were transfected with EGFP and after 16 h of expression and exposure to LPA, were fixed, and subjected to immunocytochemistry applying antibodies against EGFP and Syp I. A reduction in Syp I-positive terminals was observed in the presence of LPA. **(c)** The effect of LPA was dose-dependent, as shown by the dose response graph. **(d)** The density of neurons was unchanged in presence of LPA. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

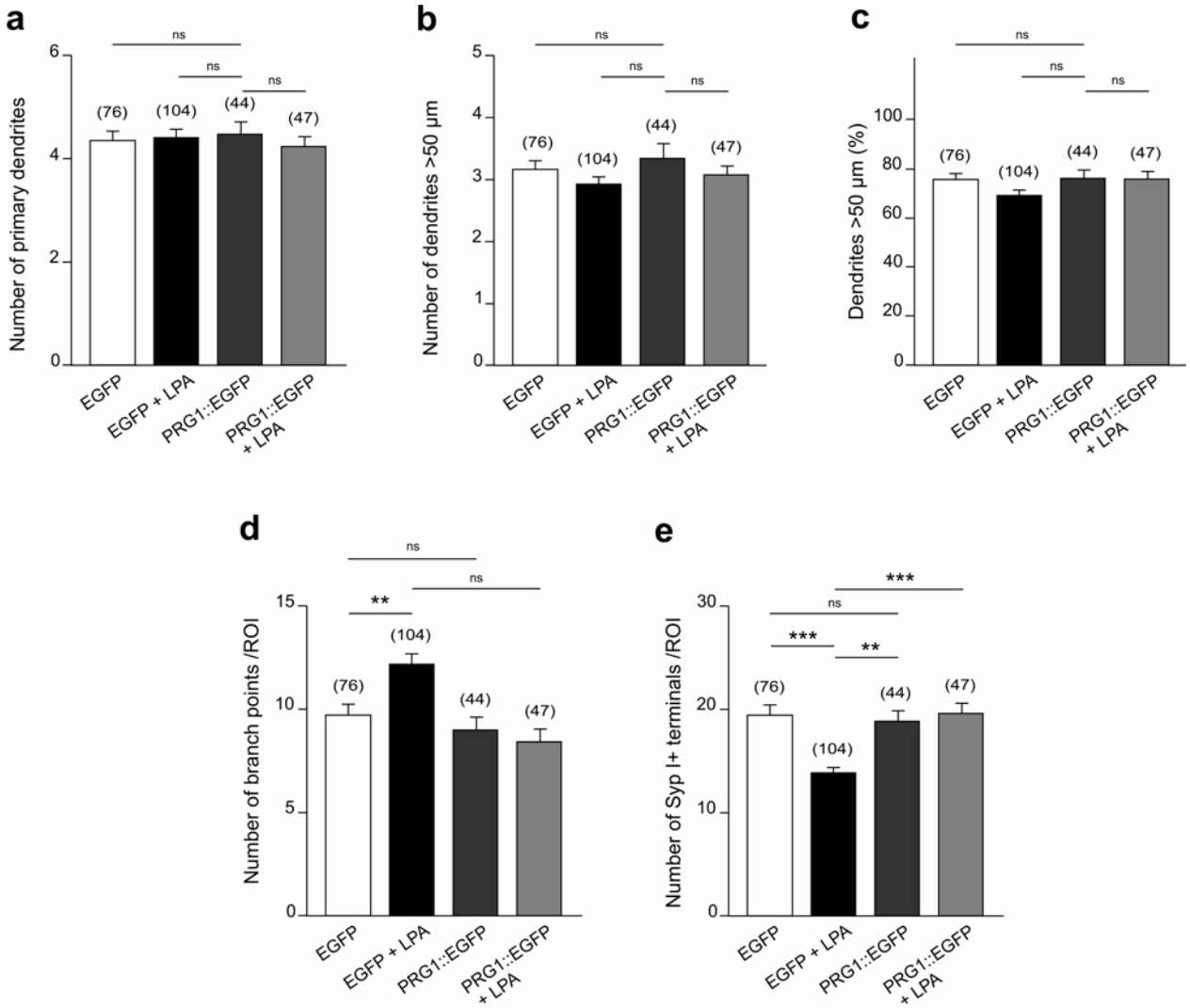


Figure 20. Quantification of the effects of overexpression of PRG-1. (a-c) No effect of LPA and overexpression of PRG-1 on primary dendrite formation and elongation. **(d)** LPA caused a slight increase in dendritic branching which was blocked by PRG-1 overexpression **(e)** PRG-1 prevented LPA-induced decrease in synapse number. Numbers in parentheses denote the number of neurons studied for the respective treatment group. Significance levels are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, ns not significant.

5.2. Synapse type-specific effects of PRG-1 on glutamatergic and GABAergic synapses

To determine whether PRG-1 overexpression had an effect on either E or I type terminals, triple immunocytochemical labeling for EGFP, VGluT1/2 and VIAAT, was performed and the number of VGluT- and VIAAT-positive spots was quantified (Fig. 21). The quantification revealed that LPA-treatment preferentially reduced the number of glutamatergic synaptic terminals (compare Fig. 22 a and b) and PRG-1 prevented this synapse loss. These results support the idea that PRG-1 signaling is relevant for synapse stabilization.

5.3. Suppression of PRG-1 expression results in synapse loss

To evaluate the consequences of reduced PRG-1 expression, the technique of RNA interference (Elbashir et al., 2001) was applied. An expression vector for small-hairpin RNA (small-interference RNA, siRNA) targeted against the rodent PRG-1 was cotransfected with the PRG1::EGFP construct on DIV 5. The knock-down effect of the siRNA was checked prior to the experiments, using Western blot from cell lysates (Bräuer, unpublished results) showing a more than 90% block after two days. The cells were analyzed two days after transfection and it was found that, despite the induced overexpression of PRG-1, cotransfection with the siRNA against PRG-1 significantly reduced the number of VGluT1/2-positive terminals both in the presence and absence of LPA (Fig. 23a). Even VIAAT-positive terminals, typically more resistant to LPA-challenge, started to disappear after PRG-1 suppression (Fig. 23b). This is a strong evidence for a role of PRG-1 in synapse stabilization. So, this siRNA was strongly functional not only against endogenous PRG-1, but also against the overexpressed PRG-1. However, experiments with a control siRNA, and knock-down of endogenous levels of PRG-1 will need to be performed to further validate the data.

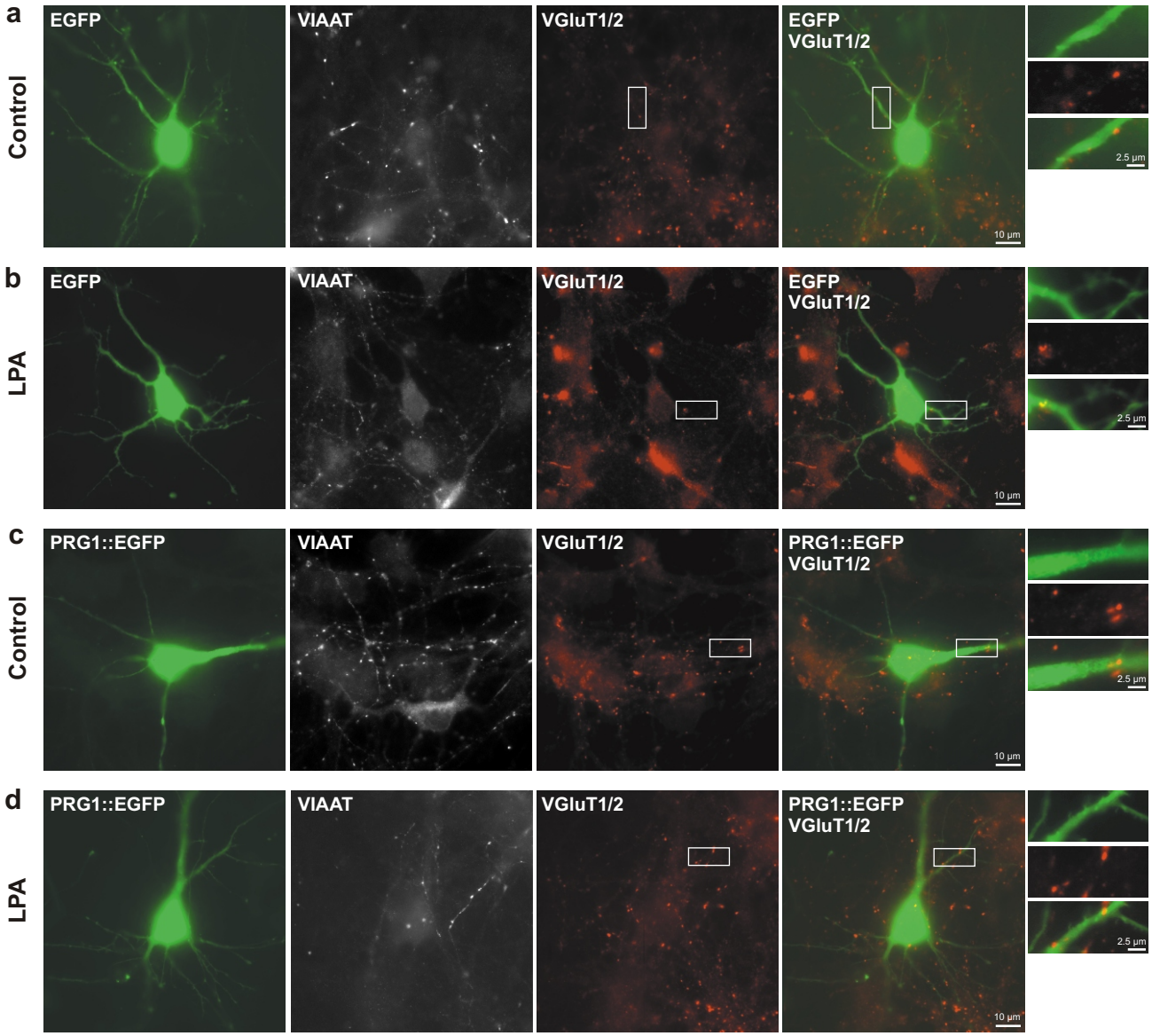


Figure 21. Immunofluorescence images of primary hippocampal neurons transfected with EGFP or PRG1::EGFP. On DIV 5, neurons were transfected and after 16 h of expression and exposure to LPA (10 µM) were fixed. Triple immunostaining was performed for EGFP, (image pseudocolored as green), VIAAT (black and white) and VGluT1/2 (image pseudocolored as red). The fourth image is the overlay of the green and red corresponding images. Boxed area is magnified on the right. **(a)** A representative EGFP-transfected neuron. **(b)** An EGFP-transfected neuron treated with LPA. A reduction in the number of VGluT1/2-positive (VGluT1/2+), but not VIAAT+ synaptic terminals was found after LPA-treatment. **(c)** A PRG1::EGFP transfected neuron from untreated group. **(d)** A PRG1::EGFP-transfected neuron from the LPA-treatment group.

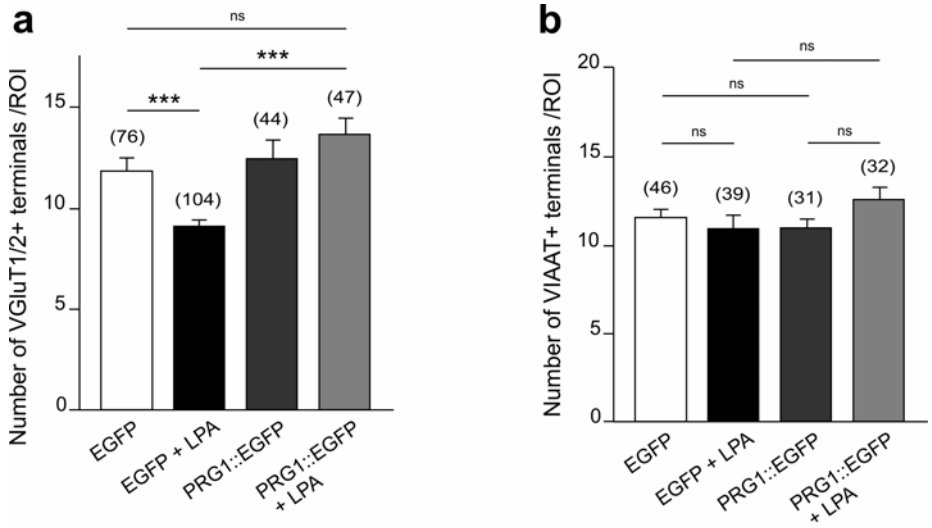


Figure 22. Synapse-type specific effects of PRG-1 and LPA. (a, b) LPA reduced the number of glutamatergic, i.e., VGluT1/2+ (a) but not the number of GABAergic, i.e., VIAAT+ (b) synaptic terminals. Overexpression of PRG-1 attenuated the LPA-mediated preferential reduction of glutamatergic terminal numbers.

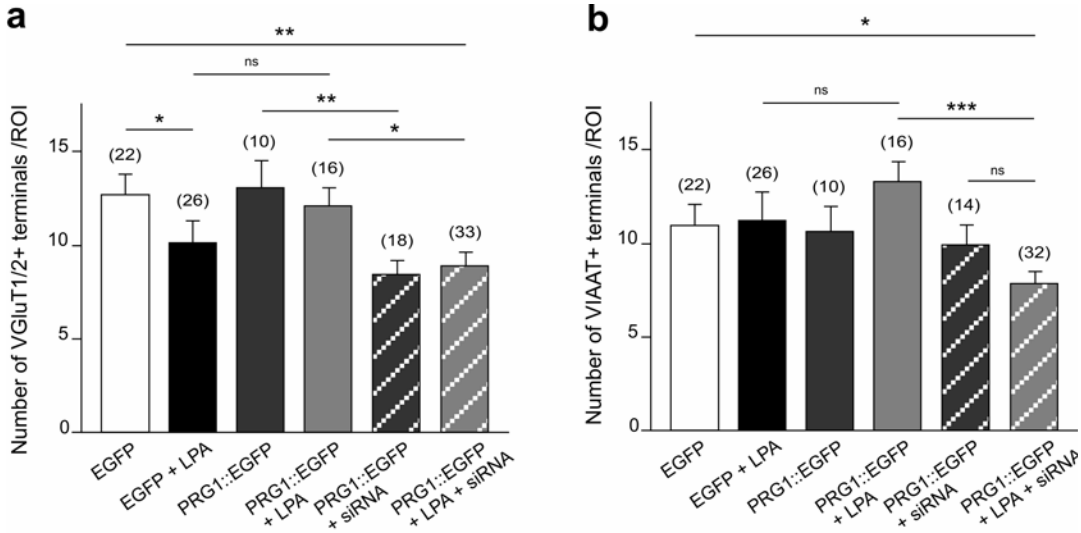


Figure 23. Silencing of gene expression of PRG-1 reduces synapse formation. Plasmids encoding for small interference RNA (siRNA) targeted against PRG-1, and PRG1::EGFP were cotransfected in hippocampal neurons on DIV 5 and allowed for expression for 48 h. **(a)** Cotransfection of siRNA and PRG1::EGFP resulted in a reduced number of glutamatergic VGluT1/2+ synaptic terminals both in the presence and absence of LPA. **(b)** Cotranfection also caused a decrease in the number of GABAergic VIAAT+ synaptic terminals, but only in the presence of LPA indicating that PRG-1 is essential for synapse formation and/or stability.