

MATERIALS AND METHODS

1. MATERIALS

1.1. Chemicals

The Neurobasal-A cell culture medium (unless stated otherwise) as well as phosphate-buffered saline, L-glutamine, mixture of antibiotics, B27 supplement (which contained no BDNF) and minimum essential medium (MEM) were purchased from Gibco BRL Life Technologies (Karlsruhe, Germany). Other chemicals used for cell culture, such as, trypsin, ovomucoid (trypsin inhibitor), ethylene diamine tetra acetic acid (EDTA), beta-mercaptoethanol (β -ME), deoxyribonuclease (DNAse) type IV, bovine serum albumin (BSA), poly-L-ornithine (PO) and agar were purchased from Sigma (Munich, Germany). Chemicals, namely, paraformaldehyde (PAF), ammonium chloride, Triton X-100, agarose and diethylpyrocarbonate (DEPC) were also from Sigma. Vectashield (with DAPI) was from Vector labs (CA, USA). Gelatine and magnesium sulphate were from Merck (Darmstadt, Germany). Neurotrophins namely, BDNF, NGF and NT-3 were from Alomone (Jerusalem, Israel). Diethyl ether used to anesthetize mice was from Hoechst (Frankfurt, Germany). Lysophosphatidic acid (LPA) was from Sigma (Plymouth Meeting, PA, USA). Proteinase K, ethidium bromide, N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES) and sodium dodecyl sulphate (SDS) were from Roth (Karlsruhe, Germany). Fetal calf serum (FCS), Trizol reagent and Superscript II reverse transcriptase were from Invitrogen (Karlsruhe, Germany). The transfection kit with the effectene reagent and the maxi-prep kit were from Qiagen (Hilden, Germany). The rapid DNA Ligation kit was from Roche Diagnostics GmbH (Mannheim, Germany). The matrix Gel Extraction System was from Marligen Biosciences, BioCat GmbH (Heidelberg, Germany). *RedTaq* DNA polymerase, 10x buffer with Mg and dNTP-mix were purchased from Sigma. All other standard laboratory chemicals were either from Merck, Roth or Sigma.

1.2. Solutions

Phosphate-buffered saline-calcium and magnesium free (PBS-CMF): 137.0 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 , 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 mM, Glucose, 15 mM HEPES and antibiotics mixture (100x) diluted to give 5 $\mu\text{g}/\text{ml}$ penicillin, 12.5 ng/ml streptomycin.

Standard salt solution (SSS): 137.0 mM NaCl, 5 mM KCl, 1.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mM NaHCO_3 , 25 mM Glucose. H_2O , 15 mM HEPES and antibiotics mixture (100X) diluted to give 5 $\mu\text{g}/\text{ml}$ penicillin, 12.5 ng/ml streptomycin.

Phosphate-buffered saline with EDTA (PBS-CMF-EDTA): 1.0 mM EDTA, 137.0 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4 , 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 mM Glucose, 15 mM HEPES and antibiotics mixture (100x) diluted to give 5 $\mu\text{g}/\text{ml}$ penicillin, 12.5 ng/ml streptomycin.

Trypsin 0.5% or 0.012%: freshly prepared in PBS-CMF-EDTA.

Poly-L-ornithine 0.005%: in sterile dH_2O .

DNase/ovomuroid: 25 mM MEM-HEPES, 5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1380 U/ml (0.06%) DNase and 5 mM ovomucoid.

Neurobasal with supplements: Neurobasal medium, 2% B27 supplement, 12.5 μM beta-mercaptoethanol, 250 μM L-glutamine, 1% FCS and antibiotics mixture (100x) diluted to give 5 $\mu\text{g}/\text{ml}$ penicillin, 12.5 ng/ml streptomycin. pH 7.3 - 7.5, osmolarity 300 mOsm/l.

Ammonium chloride: 50 mM NH_4Cl in PBS 1x

PBS-gelatine: 1.25% gelatine in PBS

Triton X-100: 0.012% Triton X-100 in PBS-gelatin

Tail lysis buffer: 1 mM Tris-HCl (Tris dissolved in 1M HCl, 12.11 g/100 ml), 0.1 M EDTA, 0.5% SDS and 0.02% RNase A.

Tris-EDTA (TE) buffer (pH 8.0): 10 mM Tris-HCl and 1 mM EDTA.

DNA solubilizing buffer (pH 8.5): 5 mM Tris-HCl

Tris-acetate-EDTA (TAE) buffer (pH 8.0) (10X stock solution): 400 mM Tris-acetate and 10 mM EDTA. To obtain the working solution, the stock solution was diluted 1:10 with ddH₂O.

SYBR Green PCR Master Mix: The SYBR Green PCR Master Mix contained SYBR Green I Dye, AmpliTaq Gold™ DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer components. For 20 µl reaction volume, the master mix was prepared as follows: SYBR Green PCR Master Mix (2X) 10 µl, forward primer (10 µM) 0.5 µl, reverse primer (10 µM) 0.5 µl, nuclease free water 6 µl and sample cDNA 3 µl.

1.3. Primers and restriction enzymes

Primers for BDNF gene sequence amplification were purchased from BioteZ Berlin-Buch GmbH (Berlin, Germany) and their 5' to 3' sequence is the following:

BDNF::EGFP antisense

5' GGG GGA TCC GAT CTT CCT CTT TTA ATG GTC AGT GTA C 3'

BDNF::EGFP sense

5' GGG GAA TTC TAC TTT GAC AAG TAG TGA CTG 3'

The primers for genotyping *bdnf* knock-out mice were also purchased from BioteZ. The sequence (5'-3') of the three primers used was the following:

Primer BD-2A for *bdnf* heterozygous +/-

5' GTG TCT ATC CTT ATG AAT CGC 3'

Primer BKO-1 for *bdnf* wild-type homozygous +/+

5' ATA AGG ACG CGG ACT TGT ACA 3'

Primer 3'-neo for *bdnf* knock-out homozygous -/-

5' GAT TCG CAG CGC ATC GCC TT 3'

The restriction enzyme *Bam*HI was from New England Biolabs, Beverly, MA, USA and *Eco*RI was from Promega, Mannheim, Germany.

1.4. Plasmids and cDNA

The EGFP-N1 vector, used for preparing BDNF::EGFP construct was from Clontech, CA, USA. PRG1::EGFP and the plasmid construct encoding siRNA against PRG-1 (Super 848) were gifted by Anja Bräuer, Institute of Anatomy, Charité (Berlin, Germany).

1.5. Competent cells

The JM-109 strain of *E.coli*, from Promega (Mannheim, Germany), was used for sub-cloning and transformation.

1.6. Antibodies and blocking agents

The Trk receptor blocking agent, K252a was from Sigma and the p75 blocking antibody was from Advanced Targeting Systems (San Diego, CA, USA). The NMDA receptor blocker, MK801, was from Merck. (S)-MCPG, an antagonist of type I and II metabotropic glutamate receptors and DNQX, a non-NMDA glutamate receptor antagonist were from Tocris (Cologne, Germany). The complete list of the antibodies used in the experiments is detailed in Table 1.

1.7. Consumables and special appliances

Pipettes and reaction tubes were purchased from Eppendorf (Hamburg, Germany). Cell culture dishes (3.5 cm) were from Greiner Labortechnik (Gloucestershire, UK). All glassware was from Scott except glass-slides which were bought from Roth (Karlsruhe, Germany). Filter tips were from Sorenson Bioscience (Salt Lake City, UT, USA) and filters used for sterile filtration were from Neolab (Heidelberg, Germany). Syringes were from Becton Dickinson GmbH (Heidelberg, Germany). 24-well plates were from Techno Plastic Products (Trasadingen, Switzerland). The Fuchs Rosenthal chamber for cell counts was from Assistent (Sondheim, Germany). The ultra centrifuge (model Biofuge pico) was from Heraeus Instruments (Langenselbold, Germany). The PCR machine (model PCR Sprint) was from

Hybaid GmbH (Heidelberg, Germany). The CO₂ incubator (model NUAIRE) was from ZAPF Instruments (Sarstedt, Germany). The Agarose gel electrophoresis system was from BioRad (Munich, Germany). The Fluorescent microscope Axiovert was from Carl Zeiss (Oberkochen, Germany). Filter-sets namely XF3017, XF32, XF110-2 and XF136-2 for detection of fluorescent signal from fluorophores FITC, Cy3, Cy5 and Alexa Blue respectively were purchased from Omega Optical Inc. (Brattleboro, VT).

1.8. Software

PMIS 4.1.4 from Remington, Munich, Germany, was the software used for image acquisition. Image overlays were made using Adobe photoshop 5.0. Sigmaplot version 8.0 was used for plotting graphs and performing statistical tests. For quantitative real-time RT-PCR, primers were designed using Primer Express 2.0. ABI Prism 7000 Sequence Detector version 1.0 from Applied Biosystems, Weiterstadt, Germany, was used for running the PCR program. NeMo, an in-house software developed by Dr. Henneberger (Institute for Physiology, Charité, Berlin, Germany) was used for verification of synaptic terminals.

1.9. Animals

bdnf knock out mice were generated in our laboratory as described by Ernfors et al., 1994. Briefly, these mice were raised from two pairs of *bdnf*^{+/-} mice in C57BL/6 background (Ernfors et al., 1994), purchased from Jackson Laboratories (Bar Harbor, ME, USA). These mice have a low rate of pregnancy and a small number of pups per litter. The nursing females are poor caretakers of their pups, resulting in a high rate of mortality, particularly for homozygotes. To raise a larger number of mice necessary for our experiments, a new line of mice was generated by crossing a male BL/6/BDNF ^{+/-} with a CD1 female mouse. The F1 ^{+/-} mice were crossed among themselves. The F2 mice were then used as founders to generate all of the mice used in the present study (referred to as CD1/BDNF mice). The *bdnf*^{-/-} mouse pups were usually smaller than the ^{+/+} and ^{+/-} littermates (Rothe et al., 1999) and died within one month after birth. They also showed defects in coordination of movement and balance, ataxia, spinning during periods of hyperactivity, and recurrent episodes of freezing seizures. Heterozygous mice were fertile and showed no overt abnormalities. For PRG-1

overexpression in hippocampal or entorhinal cortical cultures, we used E18 embryos from C57BL/6 mice.

Table 1. List of antibodies.

| | Antibodies | Donor | Dilution | Supplier |
|-----------------------------|------------------------------------|--------------|-----------------|------------------------------|
| <i>Primary antibodies</i> | | | | |
| 1. | Polyclonal anti MAP2 IgG1 | mouse | 1:400 | Sigma |
| 2. | Polyclonal anti GFP IgG | rabbit | 1:100 | Molecular probes |
| 3. | Polyclonal anti GFP | chicken | 1:500 | Chemicon |
| 4. | Monoclonal anti GFP IgG2a | mouse | 1:100 | BD Biosciences |
| 5. | Monoclonal anti synaptophysin IgG1 | mouse | 1:50 | Progene |
| 6. | Polyclonal anti synapsin | rabbit | 1:200 | Chemicon |
| 7. | Polyclonal anti VGluT1 IgG | guinea pig | 1:800 | Chemicon |
| 8. | Polyclonal anti VGluT2 IgG | guinea pig | 1:800 | Chemicon |
| 9. | Polyclonal anti VIAAT | rabbit | 1:500 | Synaptic Systems |
| 10. | Polyclonal anti p75 | rabbit | 1:500 | Advanced Targeting Systems |
| 11. | Anti TrkB function blocking IgG1 | mouse | 1:500 | BD Transduction Laboratories |
| <i>Secondary antibodies</i> | | | | |
| 12. | Anti mouse IgG-Cy5 | goat | 1:200 | Dianova, Hamburg, Germany |
| 13. | Anti mouse IgG-FITC | donkey | 1:200 | Dianova |
| 14. | Anti mouse IgG-Cy3 | goat | 1:300 | Dianova |
| 15. | Anti rabbit IgG-FITC | sheep | 1:100 | Dianova |
| 16. | Anti rabbit IgG-TRITC | donkey | 1:100 | Dianova |
| 17. | Anti rabbit IgG-Cy3 | donkey | 1:200 | Dianova |
| 18. | Anti chicken IgG-FITC | donkey | 1:500 | Dianova |
| 19. | Anti guinea pig IgG-Cy5 | donkey | 1:200 | Dianova |

2. METHODS

2.1. Genotyping

DNA isolation

Genotyping of the *bdnf* mutant mice was performed using a small tissue from the tail of the embryos (E18) or new-born mice (as described in Henneberger et al., 2000). Briefly, to each tail piece taken in an Eppendorf tube, 550 μ l of tail lysis buffer was added along with 50 μ l of proteinase-K solution (10 mg/ml solution freshly made in TE Buffer pH 8.0). It was vortexed and kept in a thermoblock for 2 h at 56°C. The tail tissue was vortexed every 15-20 min. Then, it was centrifuged at 13000 rpm for 10 min at RT. After centrifugation, 500 μ l supernatant was aspirated and added to 500 μ l isopropanol. DNA was precipitated by inverting the tubes 2-3 times and centrifuged at 13000 rpm, for 1 min at RT. The pellet was again centrifuged with 1 ml of ice-cold ethanol (70%) at 13000 rpm, for 10 min at RT. After air-drying the pellet, it was dissolved in 50 μ l of DNA solubilizing buffer and kept at 65°C for 5 min. The solution was mixed by trituration and was used for PCR.

Polymerase chain reaction (PCR)

PCR was performed in 50 μ l reaction volume using 1 μ l (about 250 ng) of the isolated DNA (1 μ l DNA + 49 μ l mastermix). A 1x master-mix for one sample was prepared by mixing 5 μ l of 10x buffer+Mg, 1 μ l of dNTP mix, 1 μ l each of primers BKO-1 and 3'-neo, 2 μ l of primer BD-2A and 0.75 μ l of *RedTaq* DNA polymerase. The volume was adjusted to 50 μ l by adding ddH₂O. The PCR conditions (PCRSprint™, Hybaid-AGS, Heidelberg, Germany) were: 94°C (melting) for 5 min, 58.5°C (annealing) for 30 sec, 72°C (extension) for 60 sec. Cycling was followed by a final extension step at 72°C for 5 min. Reaction products were run on 2% agarose gel (15 μ l /well) in TAE buffer at 80 V, for 25 min at RT and visualized using ethidium bromide.

2.2. Plasmid preparation

The BDNF::EGFP construct was prepared as described in Haubensak et al., (1998). The EGFP-N1 vector was a 4.7 kb plasmid containing a multiple cloning site where the BDNF gene sequence was ligated after digesting both the vector and the insert with *EcoRI* and *BamHI* restriction endonucleases.

cDNA amplification

The template (0.5 μ l) for amplifying the BDNF gene sequence was taken from cDNA prepared from superior colliculus of P1 and P20 mice (from Jan Walter, Charité, Berlin). The complete sequence of mouse prepro BDNF cDNA (NCBI accession no. X55573) was amplified using a 5' primer which introduced an *Eco*RI cleavage site at the N terminus and the 3' primer which introduced a *Bam*HI restriction site at the C terminus of the mouse BDNF. The PCR was performed using the above-mentioned BDNF::GFP sense/antisense oligonucleotides, and run for 30 cycles, each of which was as follows: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min. Both the samples generated one 0.8 kb band each, on agarose gel (1 %) after PCR. BDNF cDNA was eluted by gel purification method using Marligen kit which is an anion-exchange-based method for DNA purification. The DNA pellet obtained was dissolved in 5 μ l of TE buffer.

Restriction digestion

5 μ l of the BDNF cDNA and 4 μ g of pEGFP-N1 vector were digested with restriction enzymes *Eco*RI and *Bam*HI for 1 h at 37°C water bath.

Ligation

Purification of bands yielded cDNA that was then dissolved in TE buffer, 3 μ l in case of BDNF and 15 μ l for EGFP-N1 vector. Ligation was done using BDNF insert in a three-fold molar excess of the vector. Rapid DNA ligation kit, Roche Diagnostics, was used for this purpose. The ligation reaction mixture was prepared as follows: 0.1 μ l EGFP-N1 vector, 2 μ l BDNF insert, 1 μ l DNA dilution buffer 5x, 5 μ l DNA ligation buffer 2x and 0.5 μ l T4 DNA ligase. The reaction mixture was incubated for 10 min at RT.

Transformation

To select the correct construct, that is, the desired BDNF::EGFP chimera, bacterial transformation was performed. Competent cells, JM 109, were thawed on ice. 5 μ l of the ligated product was incubated with 50 μ l of competent cells for 10 min on ice. A heat shock for 45 sec in a water bath at 42 °C was given, followed by a cold shock on ice for 2 min. 450 μ l of LB medium was added to the bacterial cells which were then incubated in a shaker

incubator at 37°C, 250 rpm for 1 h and spun at 6,000 rpm for 1-1.5 min at RT. 450 µl of the supernatant was discarded and the cells were re-suspended in the rest of the volume (50 µl), which was used for plating. Pre-incubated (at 37°C for 0.5-1 h) kanamycin-agar plates (kanamycin 40 µg/µl) were used. Plating rods were prepared by flaming a Pasteur pipette. After plating, the plates were incubated at 37°C overnight. Next day, a test for the transformed colonies was performed by sub-culturing as follows: single colonies were picked and were used to inoculate 1.5 ml of selection medium (LB broth with 40 µg/µl kanamycin). The tubes were incubated for 6-8 h at 250 rpm at 37°C.

Miniprep for four clones

1 ml of each mini-culture was centrifuged for 10 seconds in 1.5 ml microcentrifuge tubes. From each of the samples, 850 µl of supernatant was removed, and the remaining supernatant was used to resuspend the bacterial pellets. 200 µl of 0.2 N NaOH/1% SDS was added to each of the samples and mixed by inverting the samples 4-5 times. Then, 150 µl of 3M potassium acetate-2M acetic acid was added and inverted again 4-5 times and microcentrifuged for 1 min. The sample supernatants were ethanol-precipitated for 2 min at RT, followed by 1 min recentrifugation. The DNA pellet of each sample was washed with 1 ml of 80% ethanol and air-dried, followed by resuspension in freshly filtered sterile ddH₂O (10 µl). To confirm that the colonies contained the BDNF::EGFP construct, restriction digestion was performed using *EcoRI* (1 U) and *BamHI* (1 U), at 37°C for 1 h. All the four clones were found to be positive for the BDNF::EGFP construct. Two of them were further checked by sequencing at Invitex (Berlin-Buch, Germany) and then used for transforming large cultures for obtaining the amplified clones.

Preparation of large cultures

In a 500 ml conical flask, 200 ml of LB medium containing kanamycin (40 µg/µl) were added. This medium was inoculated with 1 ml of the sub-culture and incubated at 250 rpm in a shaker incubator at 37°C overnight.

Maxiprep for two clones

This procedure was followed by using Endofree Maxi Kit, Qiagen, Hilden, Germany. After 12 h of growth, the bacterial cells were harvested by centrifugation at 6,000 x g for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml of buffer P1 with RNase A. To lyse the cells, 10 ml of buffer P2 was added, and the suspension was mixed by inverting 4-6 times and incubated at RT for 5 min. During the incubation, the QIA filter cartridge was prepared by screwing the cap onto the nozzle of the cartridge, which was then placed in a 50 ml Falcon tube. After 5 min, 10 ml of chilled buffer P3 was added to the lysate and mixed by inverting 4-6 times to precipitate genomic DNA, proteins and cell debris. The lysate was immediately poured into the prepared cartridge and was incubated for initial 10 min and then filtered by inserting the plunger. Approximately 25 ml of the lysate was recovered after filtration. To this, 2.5 ml of buffer ER was added, and it was mixed by inverting the tube about 10 times and incubated on ice for 30 min. During this time, an anion-exchange resin column, the QIAGEN tip 500 was equilibrated by applying buffer QBT and allowing the column to run by gravity flow. The filtered lysate was passed through this column by gravity flow. The column was then washed twice with 30 ml of a medium-salt buffer (buffer QC) which removed remaining traces of RNA and protein. The plasmid DNA was eluted with 15 ml of a high-salt buffer (buffer QN) and precipitated with 10.5 ml of isopropanol by centrifuging at 15,000 x g for 30 min at 4°C. The pellet was washed with 5 ml of endotoxin-free 70% ethanol by centrifuging at 15,000 x g for 10 min at 4°C. The pellet was air-dried for 5-10 min and redissolved in 500 µl of endotoxin-free buffer TE. The buffers used in this procedure were provided in the maxi-prep kit.

Quantification of DNA

To determine the yield, DNA concentration was measured by UV spectrophotometry. Optical density was recorded at 260 nm from diluted sample of the obtained DNA (dilution 1:100 in ddH₂O). The average of three readings was regarded as the correct value. Calculation of DNA concentration was done according to the following relation:

$$\text{DNA concentration } (\mu\text{g}/\mu\text{l}) = (\text{A}_{260} \times \text{dilution factor} \times 50) / 1000.$$

The calculated concentration was found to be the following: BDNF::EGFP = 2.15 µg/µl, EGFP-N1 = 2.0 µg/µl

2.3. RNA isolation

On DIV 5, cell cultures were treated with BDNF with or without p75 or Trk receptor blockers. The exposure time was 16 h after which, on DIV 6, RNA was isolated from the cultures.

Homogenization

Hippocampal neurons were lysed directly in the culture dish by adding 1 ml of TRIzol reagent to the 3.5 cm diameter culture dish, scraping the cell monolayer and passing the cell lysate several times through a pipette. Lysates from two dishes were collected together.

Phase separation

The homogenized samples were incubated for 5 min on ice to permit complete dissociation of the nucleoprotein complexes. 200 μ l of chloroform was added after 5 min, and the tubes were shaken vigorously for 15 sec and incubated on ice for 2-3 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remained exclusively in the aqueous phase.

RNA precipitation

The aqueous phase was carefully transferred to a fresh tube and the RNA was precipitated by mixing with 500 μ l of isopropyl alcohol. The samples were incubated on ice for 10 min and centrifuged at 12,000 x g for 15 min at 4°C.

RNA wash and solubilization

The supernatant was removed, and the RNA pellet was washed once with 1 ml of 75% ethanol (in DEPC-water) by vortexing the tube briefly and centrifuging at 7,500 x g for 5 min at 4°C. The RNA pellet was air-dried for 5-10 min and dissolved in 20 μ l of DEPC-water.

RNA quantification

To determine the yield, RNA concentration was measured by UV spectrophotometry. The Optical density was recorded at 260 nm from a diluted sample of the obtained RNA (dilution

1:100 in ddH₂O). The average of three readings was regarded as the correct value. Calculation of RNA concentration was done according to the following relation:

$$\text{RNA concentration } (\mu\text{g}/\mu\text{l}) = (\text{A}_{260} \times \text{dilution factor} \times 40) / 1000$$

2.4. Real-time RT-PCR

Reverse transcription

cDNA synthesis was performed with 2 μg of RNA using Superscript II reverse transcriptase (Invitrogen, Germany). The program for reverse transcription reaction was 70°C for 10 min (step 1), 4°C for 2 min (step 2) and 42°C for 1 h (step 3). Appropriate volumes of sample RNA solution (2 μg) were mixed with 1 μl of oligo dT Primers (0.5 $\mu\text{g}/\mu\text{l}$) and 12 μl of DEPC H₂O. This mixture was kept in the PCR machine pre-heated at 70°C (step 1). After 10 min, as temperature changed to 4°C, 7 μl of master mix (1 μl of dNTP, 2 μl of DTT, 4 μl of 5x buffer; for one sample) was added to the tubes and allowed to incubate for 2 min (step 2). Finally, at 42°C, 1 μl of Superscript II reverse transcriptase was added and allowed to react for 1 h. The cDNA was checked by running a PCR with primers for a house-keeping gene, β -actin. The presence of single bands at 0.5 kb confirmed all the cDNA samples to be suitable for quantitative analysis.

Quantitative PCR

Quantitative PCR was performed using the ABI Prism 7000 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). TaqMan probes and primers for the control housekeeping gene, *gapdh*, were Assay-on-Demand gene expression products (Applied Biosystems). All TaqMan probes were labeled with 6-carboxy fluorescein (FAM). Real-time PCRs were performed following the supplier's instructions. The PCR mix (for one reaction, 20 μl volume) was prepared by adding 3 μl of cDNA to 10 μl of SYBR Green PCR Master Mix, 0.5 μl of each forward and backward primer (0.25 μM final concentrations for each) and 6 μl of dH₂O. PCR reaction for reference genes was carried out using the TaqMan Master Mix. The primers and probes were supplied as a mixture by the manufacturer. The PCR mix was prepared similarly as for the test genes, except that 1 μl of the primer-probe mixture was added to the reaction mixture. All reactions were performed in triplicates, and the experiments were repeated at least three times. The standard curves for *syn I*, *syp I*, *vglut2*, *viaat*, *trkB* and

gapdh were generated using serial dilutions (1:10, 1:20, 1:40 and 1:80 in ddH₂O) of cDNAs isolated from hippocampal cultures on DIV 6. Target gene expression was normalized for *gapdh* expression by using the relative standard curve method described by the manufacturer.

The 5'-3' primer sequence for the genes studied is given below:

| | |
|------------------------|------------------------|
| Syn I forward (5'-3') | TGCTACGTGCGGCAGCTA |
| Syn I reverse (5'-3') | TCTCCGTCTTGTGGCACACT |
| Syp I forward (5'-3') | TGCTACGTGCGGCAGCTA |
| Syp I reverse (5'-3') | TCTCCGTCTTGTGGCACACT |
| VGluT2 forward (5'-3') | CAAAACCGGGAATTTACGTTGT |
| VGluT2 reverse (5'-3') | TTTGGGCGGGTGCTGAT |
| VIAAT forward (5'-3') | AATTTTGGCTCCTGTCAACGA |
| VIAAT reverse (5'-3') | CACCCTCAAGGTCAAGTTTCCA |
| TrkB forward (5'-3') | GACCCAAACTCTTCGCTAACGA |
| TrkB reverse (5'-3') | CCTGGTGCTGCTCCGTTCTA |

Data analysis

The results of the amplification plot of the real-time PCR were exported to Microsoft Excel sheet where the mRNA levels were quantified according to following relation:

Threshold cycle number (C_t) = intercept + slope x log [cDNA dilution]

Therefore, \log [cDNA dilution] = (C_t - intercept)/ slope. The values for slope and intercept were obtained from the prepared standard curve for each of the gene analyzed. The relative quantity (in arbitrary units) of the mRNA was normalized to that of the house-keeping gene *gapdh*.

2.5. Cell culture

Tissue dissection and dissociation

On embryonic day 18, timed pregnant, CD1/BDNF mice (the day of pairing was considered as E0) were anesthetised with diethyl ether, and the fetuses were removed and placed in standard salt solution (SSS) on ice. The genotype of each fetus was confirmed by PCR and only *bdnf*^{-/-} offsprings were selected for experiments. The hippocampi were then dissected out, dissociated with trypsin (0.5%) for 5 min at RT. Trypsinization was stopped by washing the cells in DNase/ovomucoid solution containing 25 mM MEM-HEPES, 5 mM MgSO₄·7H₂O, 1380 U/ml (0.06%) DNase and 5 mM ovomucoid. Further mechanical dissociation was performed by triturating the cells in the medium, Neurobasal-A.

Plating and maintenance

Cells were plated onto poly-L-ornithine-(0.005%)-coated dishes at an initial density of 75,000 cells/cm². Hippocampal neurons were grown at 37°C/5% CO₂ using the medium Neurobasal-A supplemented with 1% FCS, 2% B27, 12.5 μM beta-mercaptoethanol, 250 μM L-glutamine, 5 μg/μl penicillin and 12.5 μg/μl streptomycin (Brewer et al., 1993). The growth medium was half exchanged with fresh medium 24 h after plating. For PRG-1 transfection studies, the hippocampi were dissected and cells were plated as described above except that trypsinization was done with 0.12% trypsin at 37°C in a water-bath for 10 min. These cultures were also maintained in Neurobasal-A with the supplements listed above.

2.6. Transfection

After 5-7 days in vitro (DIV), neurons were transfected with the following constructs: EGFP, BDNF::EGFP, PRG1::EGFP or Super848 (plasmid vector encoding siRNA against PRG-1) using the effectene reagent (Qiagen, Hilden, Germany) which is essentially a non-liposomal lipid formulation. A concentration of 2.9 ng of DNA per 1000 cells was used for transfection. The transfection solution containing the desired DNA was prepared by mixing 2.9 ng of DNA per 1000 cells with the DNA condensation buffer (buffer EC, 0.6 μl/1000 cells). Then, the supplied enhancer (0.51 μl/100 ng DNA) was added, and the mixture was vortexed at low speed for 1 s and incubated at RT. After 5 min, the effectene reagent (16 μl/100 ng DNA) was added and the samples were vortexed for 10 s. After 15 min, culture medium (350 μl/ 1.3 cm coverslip) was added to the effectene-DNA complexes. The diluted complexes were applied dropwise to the cells. The cultures were incubated with transfection complexes at 37°C, 7.5%

CO₂ incubator for 2.5 h, after which the coverslips were transferred back to the original medium. After the desired time of expression (16 h, 48 h or 72 h), the cells were fixed for immunocytochemistry.

2.7. Immunocytochemistry

After 16, 48 or 72 h of gene expression, the transfected cultures were fixed for 15 min with ice-cold paraformaldehyde (4% in 4% sucrose) at RT. The fixation time was 5 min at 37°C in PRG-1 experiments. The fixed cultures were washed thrice with PBS-CMF and incubated in NH₄Cl (50 mM) at RT for 20 min. The cells were washed again, once with PBS-CMF and once with PBS-Gelatine. Permeabilization was done with 0.12% Triton X-100 for 4 min at RT followed by washing with 0.12% PBS-gelatin. Neurons were incubated for 1 h with the primary antibodies and then washed thrice with 0.12% PBS-gelatin. Appropriate secondary antibodies conjugated to FITC, TRITC, Cy3 or Cy5, were applied for 45 min. The cultures were washed thrice with PBS-Gelatine (0.12%) followed by one washing with PBS and finally mounted in Vectashield (3 µl for 1.3 cm, 1.4 µl for 1.0 cm coverslip). The glass slides were dried and stored at -20°C until they were examined under fluorescent illumination. All steps were performed at RT, unless otherwise stated.

2.8. Fluorescence microscopy

Image acquisition and image analysis

Images were acquired using a standard Zeiss epifluorescence microscope and Zeiss oil immersion objectives 63x, NA 1.4 or 40x, NA 1.3, a 12-bit cooled CCD camera (Ch250, Photometrics, Tucson, AZ), and the software PMIS 4.1.4. Appropriate filters (XF3017, XF32, XF110-2, XF136-2) allowed the detection and separation of fluorescent signals. The acquisition area of the digital camera was set at 1035 pixels x 1035 pixels rendering the pixel size 0.1 x 0.1 µm² or exactly 0.096 µm x 0.096 µm² for 63x objective). Thus, the dimensions of each photographed image were 100 x 100 µm² as also confirmed using a calibration slide. The digital black and white images were exported as 8-bit tiff files, and overlays were generated using Adobe Photoshop 5.0 applying false colors. For example, in a triple-staining experiment for EGFP, MAP2 and synaptophysin I, the corresponding images were colored green, blue and red, respectively.

Evaluation of dendrite morphology

The transfected neurons were evaluated in a circular view field of radius 50 μm centered at the soma. Morphological assessment of dendrites was performed manually. The number of primary dendrites was obtained by counting those neurites which were MAP2 positive and which were at least 10 μm in length. Dendritic elongation was assessed by calculating the percent fraction of dendrites exceeding or reaching the view field margins. Dendrite branching per neuron was quantified by counting the number of branch points on dendrites within the view field.

Evaluation of synapse number

In the same view field, the total number of synapses was quantified by manually counting the number of Syp I- or Syn I-positive puncta in contact with the EGFP-labeled dendrites or somata. VGluT1/2-positive and VIAAT-positive puncta were counted to assess the number of glutamatergic and GABAergic synaptic terminals respectively. A labeled punctum was considered to be a synaptic terminal, if the spot area was at least 0.2 μm^2 , with a diameter between 0.5 to 2 μm and the threshold intensity being equal to the average intensity of the ROI plus three times its standard deviation. The images were thresholded using an in-house software (NeMo) for this assessment.

2.9. Data presentation and statistical analysis

Numerical data is reported as mean \pm standard error of mean (SEM), with n being the number of neurons studied for each respective group in a set of at least three similar experiments. Unpaired Student's t -test was performed for statistical comparisons among the test and the control groups, using Sigma Plot. The differences were judged statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). The level of significance and the number of neurons studied is given in brackets above the respective data point.