2 MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Neural progenitor cells grown as neurospheres

Neural progenitor cells were prepared from 20 postnatal day seven CD1 mice, all from one litter (Charles River, Wilmington, MA, USA). Animals were sacrificed and whole brains were removed. The caudal part, including the hippocampus, and the frontal part, including the olfactory bulb, were removed by two coronal cuts (Fig. 7 A). The resulting tissue block was layed on the posterior surface. Two parasagittal cuts just peripheral to the lateral ventricles and two horizontal cuts at about the level of the corpus callosum and below the ventricles (Fig. 7 B) were made to remove tissue. The resulting tissue fragment was placed in prewarmed preparation medium (see below). This tissue was digested with Accumax (PAA Laboratories, Coelbe, Germany) for 10 min at 37°C and fragmented with a cell strainer (40 µm, BD Falcon, Heidelberg, Germany). The cell number was determined with a CASY1 CellCounter according to the manufacturer's instructions (Schärfe System, Reutlingen, Germany). The cells were diluted in neurosphere medium to a concentration of 50,000-100,000 cells/ml. This suspension was plated in suspension cell flasks (Nunc, Wiesbaden, Germany), which have a hydrophobic surface to avoid attachment of cells. Cells were kept at 37°C in 5% CO_2. After 8 to 11 days in culture the neurospheres were separated from medium and cellular debris by centrifugation (3 min, 20 g, RT). Neurospheres were separated into single cells by incubation with Accumax, as above, followed by about 20-30 careful triturations with a fire-polished Pasteur pipette. The cells were replated at 50,000-100,000 cells/ml. Pictures of cultured cells were taken with an Axiovert 100 microscope (Zeiss, Germany) and a digital CCD camera DSC-S75 (Sony).

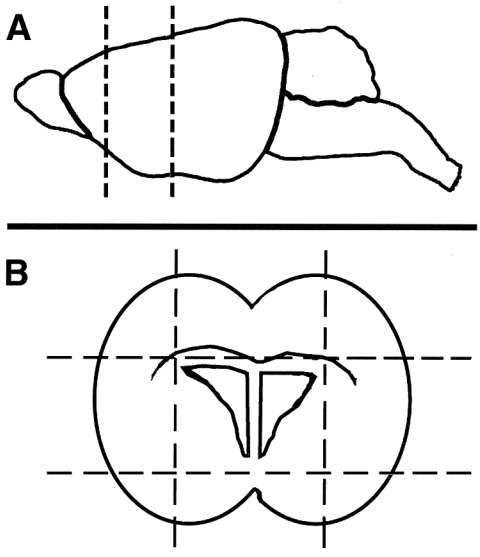

Figure 7. Schematic view of the dissection protocol. The brain was cut twice coronally between the rhinal fissure and the hippocampus (broken lines in A). The resulting tissue chunk was layed on its posterior surface. Two parasagittal cuts just peripheral to the lateral ventricles, and two horizontal cuts at about the level of the corpus callosum and below the ventricles were made (broken lines in B). Neurosphere-generating cells were extracted from the central, rectangular piece of tissue containing the lateral ventricles. Adopted from Laywell et al., 2002.

Preparation medium		Neurosphere medium	
DPBS w/o Ca and Mg (Cambrex, Verviers, Belgium)		D-MEM/F12 (1:1) with Glutamax B-27 Supplement	
Penicillin / streptomycin (Invitrogen, Karlsruhe, Germany)	100 U/ml / 100 µg/ml	HEPES	10 mM
		Insulin	20 µg/ml
Fungizone (Invitrogen)	2.5 µg/ml	Penicillin / streptomycin	100 U/ml / 100 µg/ml
D(+)-Glucose (Merck, Darmstadt, Germany)	0.6%	Fungizone	2.5 µg/ml
		Human recombinant EGF (all from Invitrogen)	20 ng/ml

2.1.2 Neural progenitor cells grown as monolayer

A litter of 12 postnatal day 8 CD1 mice was used to establish a monolayer culture of neural progenitor cells. The tissue containing the progenitor cells was isolated as described above. The cells were diluted in 'monolayer day one medium' to a concentration of 200,000 cells/ml, plated onto dishes coated with poly-L-ornithine and laminin, and incubated at 37°C in 5% CO_2. Dishes and coverslips were coated by incubation with poly-L-ornithine (15 µg/ml in H_2O, Sigma, Munich, Germany) for 1 h at 37°C, washed briefly with PBS, incubated with PBS (Cambrex) for 1 h at 37°C, briefly washed again with PBS, incubated with laminin (40 µg/ml, Sigma, diluted in D-MEM/F12) for 10 min at RT, and washed twice with D-MEM/F12.

The next day the 'monolayer day one medium' was replaced by neurosphere medium, which was supplemented with human recombinant bFGF (20 ng/ml, PAN Biotech, Aidenbach, Germany). Four days later, this medium was replaced by neurosphere medium without bFGF. The culture reached confluency after another 5 days. To passage these cells, they were incubated with Accumax for 10 min at 37°C, diluted with D-MEM/F12, centrifuged (5 min, 140 g, RT), resuspended in neurosphere medium to a density of 100,000 cells/ml, and transferred to coated dishes. The culture reached confluency again after 6 days and was treated as above. The cells were plated onto poly-L-ornithine/laminin-coated coverslips and fixed for immunofluorescence analysis after 3 days.

Monolayer day one medium	
D-MEM/F12 (1:1) with Glutamax	
Fetal bovine serum (Biochrom, Berlin, Germany)	10%
Penicillin / streptomycin	100 U/ml / 100 µg/ml
Fungizone	2.5 µg/ml
Human recombinant EGF (PAN Biotech, Aidenbach, Germany)	20 ng/ml
Human recombinant bFGF (PAN Biotech)	20 ng/ml

2.1.3 Neural progenitor cell differentiation

For differentiation of neurosphere cells, tertiary spheres (spheres after the second passage) were used after 27 days in culture. Spheres were plated onto cell culture dishes coated with poly-L-lysine (Sigma) in neurosphere medium without EGF, but with either human recombinant BDNF or NT4 (20 ng/ml, Promega, Mannheim, Germany). For immunofluorescence analysis, spheres were plated onto poly-L-lysine-coated coverslips. Dishes and coverslips were coated with poly-L-lysine by incubation with poly-L-lysine (0.1% w/v, Sigma, diluted 1:10 in H_2O) for 5 min, followed by three washes with PBS.

For differentiation in Matrigel, spheres were resuspended in ice-cold Matrigel (200 µl, BD Biosciences, Heidelberg, Germany) diluted with 75 µl L15-medium (Sigma), and this mixture was spread onto coverslips. After incubation at 37°C in 5% CO_2 for 30 min, Matrigel medium was added, and cells were incubated for 3 days at 37°C in 5 % CO_2.

Matrigel medium	
Neurobasal Medium w/o Glutamin (Invitrogen)	
B-27 Supplement	
HEPES	10 mM
Insulin	20 µg/ml
Penicillin / streptomycin	100 U/ml / 100 µg/ml
Fungizone	2.5 µg/ml
L-Glutamine (Cambrex)	2 mM
Human recombinant EGF	20 ng/ml

2.1.4 NIH/3T3 fibroblasts

NIH/3T3 murine embryonic fibroblasts (American Type Culture Collection, Manassas, VA, USA) were kept in NIH/3T3 medium composed of D-MEM with 4.5 g/l glucose (Biochrom) supplemented with 10% fetal bovine serum, 1 mM UltraGlutamine I (Cambrex), 100 U/ml penicillin, and 100 µg/ml streptomycin. They were passaged at confluency by removing the medium, washing the adherent cells with PBS, incubating them with trypsin-EDTA (0.5 g/l trypsin, 0.2 g/l Versene (EDTA), Cambrex) at 37°C for 3 min, stopping the reaction with serum-containing medium, and plating them at a 1:10 dilution.

For cryostocks, fibroblast cells from a 75 cm^2 flask were harvested by trypsin/EDTA treatment. The reaction was stopped by addition of medium and the cells were pelleted by centrifugation (220 g, 5 min, RT). Cells were resuspended in 1.5 ml DMEM with 4.5 g/l glucose (Biochrom) supplemented with 20% fetal bovine serum and 10% 2-Methylbenzyl chloride (DMSO, Sigma), and immediately transferred to a cryotube, which was placed in a freezing container (Cryo 1°C, Nalgene, Neerijse, Belgium) filled with pre-cooled isopropanol. The

container was kept at -80°C overnight to ensure a 1°C/minute cooling rate, which is required for cell cryopreservation and recovery. Later, the tubes were transferred to a liquid nitrogen tank (Taylor-Wharton, Husum, Germany) for long-term storage.

The cells were thawed by rapid defrosting in a 37°C water bath and rapidly diluted with 10 ml medium to prevent DMSO from damaging the cells. Subsequently, the cells were collected by centrifugation (220 g, 5 min, RT) and plated out in 15 ml NIH/3T3 medium in a 75 cm^2 flask.

2.1.5 NIH/3T3 fibroblast cells stably expressing *Ptpns1* or *Cd47*

Plasmid vectors containing murine *Ptpns1* or *Cd47* under control of the constitutively active promotor P_{CMV} were constructed as described (see section 2.3.7). Plasmid DNA was isolated from *Escherichia coli* with the Qiagen plasmid maxi kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). 30 µg of each vector were linearized by digestion with the restriction enzyme *Ssp*I (New England Biolabs, Frankfurt, Germany). The DNA was precipitated with ethanol (see section 2.2.1) and resuspended in water. Complete linearization was verified by agarose gel electrophoresis (see section 2.3.1) and the DNA concentration was measured spectrophotometrically.

NIH/3T3 fibroblasts were plated at a concentration of 32,000 cells/cm^2. They were transfected using Lipofectamine 2000 (Invitrogen) according to the supplier's instructions. Briefly, DNA and Lipofectamine were independently diluted in OPTI-MEM (Invitrogen), mixed, and incubated for 20 min at RT. Then these solutions were added to the cells, which were kept in NIH/3T3 medium without antibiotics. After 4 h, the medium containing the transfection mix was removed and cells were allowed to recover in fresh medium without antibiotics. The cells were harvested from the dish by trypsin digestion the next day and replated at 3,000 cells/cm^2 in peel-off flasks (Biochrom). After another day (48 h after transfection), 1 mg/ml zeocin (Invitrogen) was added to the medium to select for clones carrying a stable insertion of plasmid DNA in the genome. Another 4 days later the medium was replaced with fresh NIH/3T3 medium containing 1 mg/ml zeocin. Almost all cells were dead after 2 more days. Only a few colonies of zeocin-resistant clones were present. These were harvested by trypsin digestion, diluted in fresh medium containing zeocin, and plated out in 96-well plates. After 8 days, the medium was refreshed. Isolated colonies that appeared to result from a single cell were expanded. A cryostock was generated of each clone, as described above. To screen for cell clones expressing the respective transgene, samples were analyzed by immunoblotting and immunofluorescence. In total, 46 clones were expanded, frozen, and screened.

2.2 Microarrays and data analysis

2.2.1 Microarray production

The array used in this study contained 13,627 clones. 10,080 of them were from the arrayTAG clone set (LION Bioscience, Heidelberg, Germany). These are short, sequence-verified, murine cDNA clones, which are located close to the 3' end of the respective transcript, but do not contain poly-A sequences. 3,510 murine cDNA clones representing brain-expressed transcripts were obtained from the resource centre of the German Human Genome Project (RZPD, Berlin, Germany). In addition, 34 plant cDNA sequences and 3 murine cDNA sequences were included. LION clones were amplified with LION 3' and LION 5' primers, RZPD and plant clones with M13 forward and M13 reverse primers. The cDNA sequences were kept as plasmid inserts in *Escherichia coli* glycerol stocks in 96-well plates.

To amplify the cDNA sequences, an automated PCR setup comprising of a robotic system from Tecan (Genesis Workstation 200, Tecan, Crailsheim, Germany) combined with a temperature-controlled hotel (automatic incubator cytomat 6002, Heraeus, Hanau, Germany) was used. Template 96-well microtiter plates containing bacterial clones as well as empty 96-well PCR microtiter plates were stored in the hotel (Fig. 8 b, c). The process setup was as follows: the respective microtiter plates from the hotel were placed onto the robotic platform (Fig. 8 a) by means of an elevator within the hotel and the robot's plate gripper. Next, PCR master mix (50 µl/well) was distributed and bacterial template (2 µl/well) was added by a liquid handling system (Fig. 8 a). The finished plates were transported back into the hotel (Fig. 8 b, c). They could be stored there or taken out while the process continued. Ready-to-cycle PCR plates were either immediately processed using PrimusHT multiblock thermal cyclers (MWG Biotech, Munich, Germany; Fig. 8 d) or stored at -20°C until PCR amplification.

In addition, this system prepared the PCR products for evaluation by agarose gel electrophoresis and for precipitation. In detail, finished PCR microtiter plates as well as empty microtiter plates were placed in the hotel (Fig. 8 b, c). Then, each PCR plate alongside an empty microtiter plate were transported from the hotel to the robotic platform (Fig. 8 d). 5 µl of gel-loading buffer was distributed into empty microtiter plates by a liquid handling system and 5 µl of PCR product were added. All PCR products in these gel-loading plates were evaluated by agarose gel electrophoresis using the RoboSeq 4204S (MWG Biotech). To the remaining 45 µl of PCR product, precipitation mix was added (see below). The combination of the Heraeus hotel with the liquid and plate handling robotic system from Tecan and the external PCR cyclers from MWG Biotech facilitated high-throughput PCR amplifications of cDNA clones as probes for DNA microarrays.

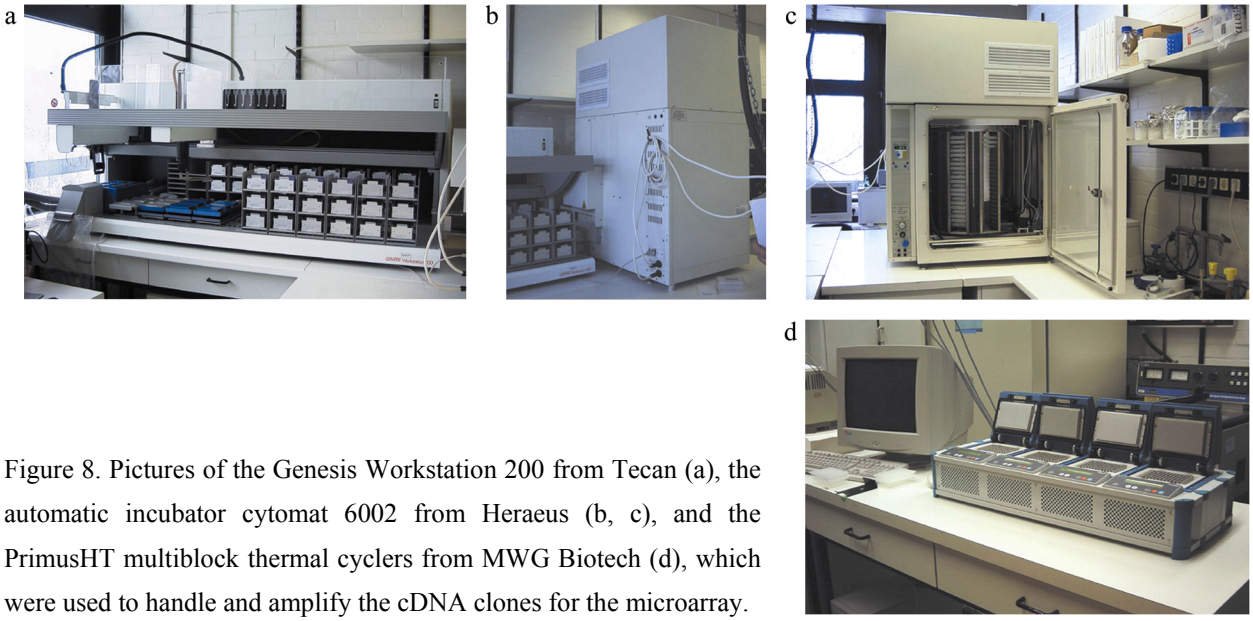

Figure 8. Pictures of the Genesis Workstation 200 from Tecan (a), the automatic incubator cytomat 6002 from Heraeus (b, c), and the PrimusHT multiblock thermal cyclers from MWG Biotech (d), which were used to handle and amplify the cDNA clones for the microarray.

PCR reaction for amplification of clones

Reaction mix		Primers	
10x PerkinElmer PCR buffer	5 µl	LION 5'	AGCGTGGTCGCGGCCGAGGT
dNTPs (1 mM each)	10 µl	LION 3'	TCGAGCGGCCGCCCGGGCAGGT
MPI Taq (made inhouse) (10 U/µl)	2 Units	M13 forward	GTAAAACGACGGCCAG
Forward primer (10 µM)	2 µl	M13 reverse	CAGGAAACAGCTATGAC
Reverse primer (10 µM)	2 µl		
Nuclease-free water	ad 50 µl		

Thermal profile	LION primers			M13 primers		
	Temperature	Time	Cycle number	Temperature	Time	Cycle number
Initial denaturation	94°C	3 min	1x	94°C	5 min	1x
Denaturation	94°C	30 sec		94°C	45 sec	
Annealing	68°C	30 sec	35x	54°C	90 sec	35x
Elongation	72°C	50 sec		72°C	2 min	
Final elongation	72°C	10 min	1x	72°C	10 min	1x

45 µl of the PCR products were mixed with precipitation solution (2.5 volumes ethanol, 0.1 volume sodium acetate), incubated at -80°C for 30 min, and centrifuged (20,000 g, 30 min, 4°C). The pellets were washed with 50 µl of 70% ethanol, centrifuged again (20,000 g, 15 min, 4°C), and resuspended in 18 µl 3×SSC. 8 µl of this solution were transferred into 384-well plates by a Multimek 96/384 (Beckman Coulter, Krefeld, Germany) and stored at –20°C. PCR products were printed onto Corning GAPS II slides by using a robotic spotting device (SDDC-2

MicroArrayer, ESI, Toronto, Canada/ChipWriter Pro, BIORAD) with SMP3 pins from TeleChem International (Sunnyvale, CA, USA). The average spot centre to centre distance was 204 µm.

2.2.2 Hybridization, washing, and scanning

For every co-hybridization, two RNA samples were labeled in an RT reaction, RNA from undifferentiated and from differentiated cells. To account for dye-specific effects, every hybridization was done twice, with the dyes exchanged by flipping the RT primers so that every cDNA was once labeled with the Cy3-specific capture sequence and once with the Cy5-specific sequence. Thus, six arrays were used for each series (NT4 and BDNF), resulting in twelve hybridizations in total.

Before hybridization, the spotted probes were rehydrated by holding the slides over hot water until a vapor coating appeared, and quickly dried by placing them on a hot plate (98°C) for 3 to 5 seconds. Then the spotted probes were crosslinked with the slide's surface by two successive UV crosslinking steps (120 mJ) in a UV Stratalinker 1800 (Stratagene, Amsterdam, The Netherlands). Remaining chemically active sites on the surface were blocked by 15 min incubation in succinic anhydride/sodium borate solution. Afterwards, the slides were briefly washed in ultrapure water and dried by centrifugation (125 g, 3 min, RT) followed by 3 to 5 seconds incubation on a hot plate (98°C). They were then ready for hybridization.

Labeling and hybridization reactions were performed using the 3DNA Array 50 Expression Array Detection Kit (Fig. 9) (Genisphere, Hatfield, PA, USA). The labeling kit was used according to the manufacturer's instructions. For each labeling reaction, 20 µg of total RNA was used. In brief, 20 µg of RNA diluted in 22 µl of ultrapure water and RT primers (1 µl of 1 pmol/µl) were mixed and heat denatured, and RNase inhibitor (1 µl) was added. Then a reaction mix consisting of 2 µl Superscript II reverse transcriptase (RT, 200 units/µl) (Invitrogen), 8 µl Superscript II reaction buffer, 2 µl dNTPs (10 mM each), and 4 µl DTT (0.1 M) was pooled with the RNA/RT primer mix. The RT enzyme was allowed to react for 2 h at 42°C before the reaction was stopped by addition of 7 µl of 0.5 M NaOH/50 mM EDTA, and the DNA/RNA hybrids were heat denatured at 65°C for 10 min. Then the reaction was neutralized by addition of 10 µl Tris-HCl (1 M, pH 7.5).

After cDNA synthesis and incorporation of the capture sequences, the cDNA was concentrated according to the manufacturer's instructions. Briefly, linear acrylamide, NaCl, and ethanol were added to the reaction. The mix was incubated at -20°C for 30 min and then centrifuged at 12,200 g at RT for 15 min. After aspiration of the supernatant, the pellets were

washed with 300 µl of 70% ethanol, recentrifuged for 5 min, and dried in a heat block at 65°C for 20 min.

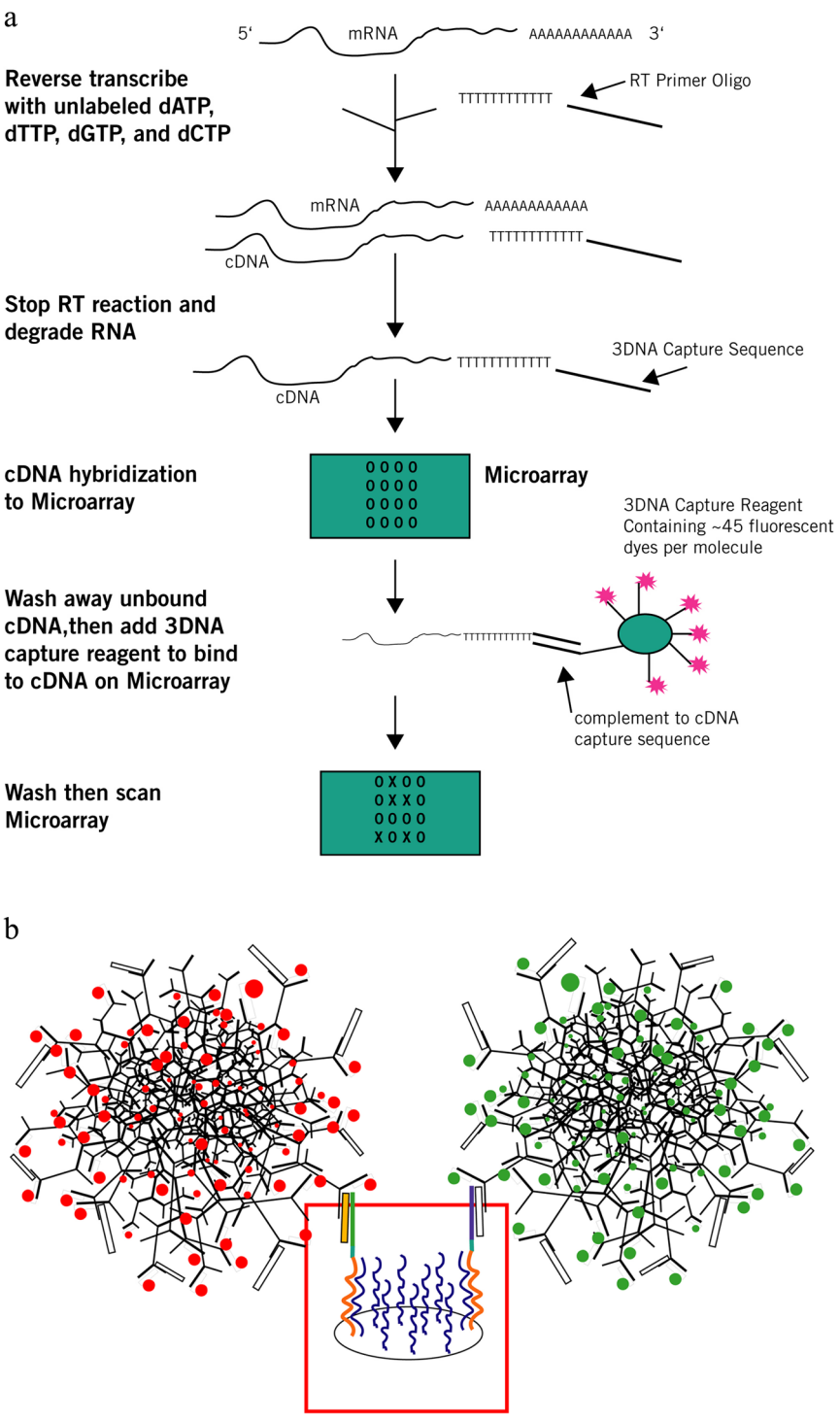

Figure 9. Labeling and detection of mRNA targets with Genisphere dendrimer technology. (a) Messenger RNA is reverse transcribed into cDNA using unlabeled nucleotides and a poly-T oligo, that carries a capture sequence. Two such cDNA populations are co-hybridized to the microarray. They are specifically detected by the 3DNA capture reagent. (b) The capture reagent contains dendrimers, large molecules composed of DNA strands coupled to fluorescent labels (Cy3 and Cy5). The dendrimers present sequences complementary to the capture sequences on the cDNA, thereby allowing to detect only cDNAs derived from one RNA pool. In consequence, a single cDNA molecule attracts approximately 45 fluorescent labels to the microarray, resulting in a higher sensitivity compared to the hybridization of cDNA labeled during the reverse-transcription with fluorescent molecules coupled to the nucleotides.

For cDNA hybridization to the array, the pellets were carefully resuspended in 10 µl of nuclease-free water and heated to 65°C for 10 min and quickly cooled on ice. Then the final hybridization mix composed of the concentrated cDNA, 2x hybridization buffer, LNA dT Blocker, nuclease free water, and Cot-1 DNA was prepared and incubated at 75°C for 10 min followed by 45°C for 20 min. Meanwhile the array was prewarmed to 45°C for 15 min in an incubator. The final hybridization mix (cDNA) was mixed, centrifuged briefly, and applied to the prewarmed array. The slide was put into a sealed humidified chamber and was incubated in a water bath at 42°C overnight.

The next day the 3DNA capture reagents containing the dendrimers were hybridized to the array. First, the array was washed by sequential incubation in 2x SSC/0.2% SDS for 10 min, in 2x SSC for 10 min, and in 0.2x SSC for 10 min at RT. To remove the remaining liquid from the slide surface, it was transferred to a slide holder and centrifuged (125 g, 3 min, RT). Meanwhile the 3DNA capture reagents were thawed at RT in the dark for 20 min. 3DNA capture reagents and the hybridization buffer were then heated to 55°C for 10 min, and an anti-fade reagent was added to the hybridization buffer. The final hybridization mix (3DNA) was prepared (see below), mixed very carefully, and incubated at 75°C for 10 min followed by an incubation at 45 °C for 20 min. The array was again prewarmed as before. The mix was applied to the array and the slides were kept in a dark humidified chamber at 42°C for 3 h. Subsequently, the array was washed as before in 2x SSC/0.2% SDS for 10 min, 2x SSC for 10 min, 0.2x SSC for 10 min, and finally briefly in deionized water. The slide was dried by centrifugation (125 g, 3 min) and incubated at 42°C for 5 min. It was stored in a dry and dark box until scanning.

Final hybridization mix (cDNA)		Final hybridization mix (3DNA)	
Concentrated cDNA	10 µl	2x hybridization buffer + Anti-Fade	25 µl
2x hybridization buffer	22 µl	3DNA capture reagent #1 (Cy3)	2.5 µl
Array 50 dT Blocker	2 µl	3DNA capture reagent #2 (Cy5)	2.5 µl
Cot-1 DNA	2 µl	Nuclease-free water	5 µl
		Cot-1 DNA	2 µl

The arrays were scanned with the Affymetrix 428 Array Scanner (Affymetrix, Santa Clara, USA). Fluorescence intensities of Cy3 and Cy5 were measured separately at 532 nm and 635 nm. The photomultiplier tube gain was typically between 40-55 dB for Cy3 and 35-45 dB for Cy5. This ensured that the signal intensity reached saturation on fewer than 1% of the spots. The resulting images were saved as 16-bit data files in tag image file format (TIFF).

2.2.3 Data processing

The image files were imported into the Microarray Suite image analysis software (Version 2.0), which runs as an extension of IPLab Spectrum Software (Scanalytics, Fairfax, VA, USA). The software determined the raw spot intensities of Cy3 and Cy5 and performed a local background subtraction. Empty spots and spots carrying plant sequences were excluded from further analysis. Each dye swap experiment was normalized by applying variance stabilization (Huber et al., 2002) using the vsn package of bioconductor (http://www.bioconductor.org). Means of normalized log-products and log-ratios of each dye swap experiment pair were used for further analysis. Normalization procedures were performed using R (http://cran.R-project.org).

To determine a meaningful cut-off value that designated differentially expressed genes, a statistical analysis was applied that considers the small number of biological replicates and calculates the percentage of false positives. To do so, a variance estimation using a pooled estimate of the variance over all genes of three self-to-self comparisons with RNA from undifferentiated cells was performed. A similar approach has been described by Sabatti et al. (2002). To achieve a robust variance measurement, the median of absolute deviation (MAD) was determined as variance estimator. The MAD in all three independent experiments of self-to-self comparison was very similar: 0.297 +/- 0.021. Based on this analysis, one can assume a rate of 2% false positives when applying a universal threshold of 2.17 fold change. A rate of 5% false positives can be assumed when applying a threshold of 1.8-fold. Therefore, all clones above a 2.0-fold change were considered relevant. Thus, the rate of false positive clones lies between 2-5% when applying this threshold.

To extract clones that are of interest for further analysis I concentrated on all clones, whose expression changed more than 2.0-fold in at least one of the three time points of differentiation in one of the experimental series, the BDNF and the NT4 series. 722 clones in the BDNF series and 624 in the NT4 series met this criterion. The intersection of both lists contained 454 clones, and only these were analyzed in greater detail to concentrate on effects common to downstream signaling of the TRKB receptor. This data filtering step further reduced the number of false positives.

2.2.4 Cluster analysis

Of the 454 clones, 441 clones showed a consistent up- or down-regulation at the three differentiation time points and were included in a cluster analysis. These 441 datasets, each representing a single time course, were clustered by applying the k-means algorithm and using a refined Euclidean distance measure. This specifically takes into account the dependence of gene

expression changes at the three time points. A k means clustering with k values ranging from 3 to 15 was performed. For our dataset, k=10 was found to be optimal to separate many clearly different dynamics without separating genes with too similar dynamics. The distance was defined as the weighted sum of k-means assignment and a similarity of shapes between cluster centers (gradient). The distance measure (D) was defined as follows:
D(x,y) = a d1(x,y)+(1-a) d2(x,y) where a = 0.5; x,y = two gene profiles to be compared; d1 the euclidean distance between x and y; and d2 the gradient of x and y. These calculations and the respective visualization were carried out using MATLAB (Version 6.0.0.88, Release12, MathWorks, Natick, MA, USA).

2.2.5 Sequencing

Whereas the clones from LION Bioscience had been sequence-verified by the supplier, the IMAGE clones from the RZPD were not. Thus, over 100 clones corresponding to differentially expressed genes were verified by sequencing. If a gene was represented by two clones on the array, that showed comparable expression changes, it was not sequence-verified. For sequencing, clones were picked from the glycerol stocks and PCR-amplified using the conditions described above. PCR products were purified with the PCR purification kit (Qiagen), evaluated by agarose gel electrophoresis, and quantified spectrophotometrically. They were sequenced using the BigDye Terminator Chemistry (PE Biosystems) with the primers used for amplification and then separated on an ABI 377 DNA sequencer.

2.3 Molecular biology

2.3.1 Agarose gel electrophoresis

DNA or RNA fragments were separated and visualized by agarose gel electrophoresis. Gels were comprised of 1% agarose (Invitrogen) in TBE buffer (0.1 M Tris, 0.1 M boric acid, 2 mM EDTA) supplemented with 0.5 µg/ml ethidium bromide. At least 0.2 volumes of gel loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol was added to the nucleic acid solutions before loading into the wells. DNA size markers (pUC mix 8 or Lambda DNA/EcoRI+HindIII 3, Fermentas, St. Leon-Rot, Germany) were also loaded. Gels were run at 100 V for 30-45 min. Nucleic acids were visualized and pictures were taken using the E.A.S.Y Win32 gel documentation system (Herolab, Wiesloch, Germany).

2.3.2 RNA isolation

Cells were mechanically removed from the dish with a cell scraper, transferred to a Falcon tube, and collected by centrifugation (220 g, 5 min, 4°C). The pellets were quickly frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated with Trizol reagent (Invitrogen). The cell pellets were resuspended in 10 ml Trizol reagent (Invitrogen) and incubated for 5 min at RT. Then 2 ml chloroform were added and the suspension was thoroughly mixed by vortexing and incubated for another 2 min at RT. After centrifugation (3,000 g, 20 min, 4°C) the upper phase was removed and divided into six aliquots of 800 µl each. These were mixed with 800 µl isopropanol, incubated for 10 min, and centrifuged (12,000 g, 10 min, 4°C) to precipitate the RNA. The pellet was washed with 500 µl 70% ethanol, centrifuged (7,500 g, 5 min, 4°C), and dried in a speed-vac. Each pellet was dissolved in 200 µl of ultrapure water. The quality was checked by agarose gel electrophoresis. Two sharp bands corresponding to 28S and 18S ribosomal RNAs and little to no background were observed. The RNA quantity was measured with a spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences, Freiburg, Germany).

2.3.3 Reverse transcription and semi-quantitative RT-PCR

2 µg of total RNA from each of the four differentiation time points were reverse-transcribed into cDNA by incubation with 5 µl dNTPs (10 mM each), 2.5 µl random primers (500 ng/µl), nuclease-free water, 10 µl M-MLV 5x reaction buffer, 2 µl RNAsin, and 800 units M-MLV RT (all from Promega) for 1 h at 37°C. PCR reactions were carried out as shown below using 1 µl cDNA from each time point as template. Annealing temperature and number of amplification cycles were specific to each transcript. PCR products were resolved and visualized by agarose gel electrophoresis.

RT-PCR

Reaction mix		Thermal profile	Temperature	Time	Cycle number
dNTPs	200 µM each	Initial denaturation	94°C	2 min	1x
Forward primer	0.6 µM				
Reverse primer	0.6 µM	Denaturation	94°C	45 sec	
Reaction buffer (Promega)	1x	Annealing	54-57°C	60 sec	21-28x
Red Taq Polymerase (Promega)	1.5 U	Elongation	72°C	90 min	
Water	ad. 24 µl				
Template cDNA	1 µl				

RT-PCR: Primers, number of cycling reactions, and annealing temperatures

Gene	Forward primer	Reverse primer	Cycle number	Annealing temperature
Igfbp2	GCGGGTACCTGTGAAAAGAG	AGCTCCTTCATGCCTGACTTG	23	57°C
Cdc2/*p34*	CAGGACTCCAGGCTGTATCTC	GCCAGTTTGATTGTTCCTTTG	23	57°C
Camk2b	GCTACCTGTCTCCCGAGGTC	GAGTAACGGTGTCCCACTCAG	24	57°C
Id4	GTCAGCAAAGTGGAGATCCTG	CTGTCACCCTGCTTGTTCAC	26	54°C
Pleiotrophin	GCAACTGGAAGAAGCAGTTTG	TGGAGATGGTGACAGTTTTCTG	22	57°C
Hb-Egf	TGATGCTGAAGCTCTTTCTGG	AGATCTGTCCCTTCCAAGTCC	23	57°C
Complement c. 3	CTGTTGCATAATCCAGCCTTC	GTATGGATGGCCACAGTTTTG	21	57°C
Txnrd1	CTAGAGCTGACTCCCGTAGCC	ATTCCAATGGCCAAAAGAAAC	25	57°C
Ptpns1 (*Shps-1*)	GTAATGTCACCCCAGCAGATG	ACACCGATGAAGACATTCCAG	25	57°C
Cd47	GGCCTCATTGTAATCTCTACGG	ATGATCCCCAAACCTGAAATC	23	57°C
Fxyd1/*Plm*	ACTATCGCTGGGATCCTCTTC	TGAGTTTCCTGGAGTCAGGTG	23	57°C
H2A.Z	CATCGACACCTGAAATCTAGGAC	ACGAGGGGTGATACGCTTTAC	21	57°C
Nell2	AAGCATACCTGCATGGAGAAC	TCCTGATGTACCCAGTTTTGC	23	57°C
6330403K07Rik	GAAGGTACCGCAAGGAGAATC	CTCTCTGGTGGTCAGTTCTGG	24	57°C
Complement c. 4	ACTTCCAGAAGGCTGTCAGTG	ACTTGCAACAGGACAAGAAGG	21	57°C
Mapk3/*Erk-1*	CCGCCATGAGAATGTTATAGG	CGGAGGATCTGGTAGAGGAAG	23	57°C
Igf1	CATGTCGTCTTCACACCTCTTC	CCAGTCTCCTCAGATCACAGC	28	57°C
Igf1 receptor	GTTATCCACGACGATGAGTGC	CTTGGAGCATTTGAGCAGAAG	24	57°C
Insulin receptor	GACCTTGGTTACCTTCTCTGATG	CTGGCCGAGTCGTCATACTC	23	57°C

2.3.4 Restriction fragment length polymorphism analysis

Neurospheres were generated from a litter of day 8 hybrid mice born after mating a female *Mus musculus* with a male *Mus spretus* (mice were provided by Markus Schülke, Charite, Berlin, Germany) as described above (see section 2.1.1). Neurospheres were cultivated and differentiated according to the standard protocol (2.1.3), and total RNA was prepared from these cells (2.3.2) Reverse transcription of RNA from undifferentiated and differentiated neurosphere cells (2.3.3) generated respective cDNAs. These served as templates for the PCR amplification of a fragment of the *Mest* transcript. The resulting PCR products were digested with *Taq*1 (Fermentas). Undigested and digested PCR products were separated by agarose gel electrophoresis (2.3.1).

PCR for amplification of a *Mest* cDNA sequence					
Reaction mix		Thermal profile	Temperature	Time	Cycle number
10x PerkinElmer PCR buffer	5 µl	Initial denaturation	94°C	5 min	1x
dNTPs (1 mM each)	10 µl				
Peg1 forward primer (10 µM)	2 µl	Denaturation	94°C	60 sec	
Peg1 reverse primer (10 µM)	2 µl	Annealing	56°C	75 sec	35x
cDNA from RT-reaction	1 µl	Elongation	72°C	90 sec	
PerkinElmer Taq polymerase	0.4 µl				
Nuclease-free water	29.6 µl	Final elongation	72°C	7 min	1x
Primers					
Peg1 forward	ATTCGCAACAATGACGGC				
Peg1 reverse	TGAGGTGGACTATTGTGTCACC				

2.3.5 Immunofluorescence of cultured cells

Neurosphere cells cultivated on coverslips were fixed by sequential incubation in ice-cold methanol (5 min) and acetone (30 sec) and stored at -20°C until use. Neurosphere cells that were used for GALC staining or differentiated in Matrigel, as well as NIH/3T3 fibroblasts were fixed in 4% paraformaldehyde/PBS. Coverslips were treated with Triton X-100 (0.1% in PBS) for 5 min (except for GALC staining on neurosphere cells and for V5, PTPNS1, and CD47 staining on NIH/3T3 fibroblasts), washed with PBS three times (5 min each), and blocked with 5% donkey serum (Dianova, Hamburg, Germany) in PBS for 30 min at RT. Primary antibodies were applied for 45 min at RT or for 16 h at 4°C. PBS without primary antibody was used as a negative control. After three washes with PBS (15 min each), secondary antibodies coupled to fluorescent dyes indocarbocyanin (Cy3) or fluorescein isothiocyanate (FITC) were applied for 30 min at RT. All secondary antibodies were from donkey and purchased from Dianova. Then the coverslips were washed again three times in PBS (15 min each), rinsed in water, dehydrated in ethanol, and air-dried. Mounting medium was applied and coverslips were inverted and placed on glass slides. The preparations were sealed with nail polish. The mounting medium contained DAPI to stain DNA and 1,4-Diazabicyclo[2.2.2]octane (DABCO) to inhibit photobleaching of fluorescent dyes. DABCO scavanges free radicals produced by excitation of fluorochromes. Immunofluorescence was documented with a Zeiss Axioskop epifluorescence microscope (Zeiss, Germany) or with a Leica DM IRE2 (Leica, Wetzlar, Germany).

PBS		Mounting medium	
Na_2HPO_4 x 2 H_2O	8 mM	Glycerol	90%
KH_2PO_4	1.5 mM	Tris-HCl, pH 8.9	0.1 M
NaCl	140 mM	DABCO	2.3%
KCl	3 mM	DAPI	0.5 µg/ml
pH 7.3		(all from Sigma)	

Primary antibodies, suppliers, applications, and the working dilutions (IF NSP: immunofluorescence on neurosphere cells, IF NIH/3T3: immunofluorescence on NIH/3T3 fibroblast cells, IHF: immunohistofluorescence on brain sections)

Primary antibody	Supplier	Application	Concentration
anti-tubulin-β-III	Upstate, Charlottesville, VA, USA	IF NSP	1:150
anti-GFAP (polyclonal)	Santa Cruz Biotechnology, Santa Cruz, CA, USA	IF NSP	1:100
anti-GFAP (MAB3402 monoclonal)	Chemicon, Temecula, CA, USA	IHF	1:100
anti-nestin	BD Biosciences, Heidelberg, Germany	IF NSP	1:100
anti-Ki-67	DakoCytomation, Hamburg, Germany	IF NSP	1:100
anti-PTPNS1	BD Biosciences	IF NSP, IHF	1:200
anti-PTPNS1	BD Biosciences	IF NIH/3T3	1:100
anti-CD47	BD Biosciences	IF NSP, IHF	1:200
anti-CD47	Santa Cruz Biotechnology	IF NIH/3T3	1:100
anti-FABP7	kindly provided by T. Mueller and C. Birchmeier, MDC, Berlin, Germany	IF NSP, IHF	1:100
anti-pleiotrophin	R&D Systems, Wiesbaden, Germany	IF NSP, IHF	1:100
anti-HUC/D	Molecular Probes, Eugene, OR, USA	IHF	1:100
anti-CD24	Santa Cruz Biotechnology	IHF	1:100
anti-PSA-NCAM	AbCys, Paris, France	IHF	1:100
anti-GALC	Roche	IF NSP	1:100
anti-V5	Invitrogen	IF NIH/3T3	1:800

2.3.6 Immunohistofluorescence

Sagittal cryostat sections (10 µm) of brains from 3 month old CD1 mice were placed on glass slides, air-dried for 30 min at RT, fixed in -20°C cold acetone for 10 min, and briefly air-dried again. If not used immediately, slides were stored at -80°C until use. Sections were treated with Triton X-100 (0.1% in PBS) for 5 min, washed with PBS three times (5 min each) and blocked with 5% donkey serum in PBS for 30 min at RT. Primary antibodies were applied for 45 min at RT or for 16 h at 4°C. PBS without primary antibody was used as a negative control. After three washes in PBS (15 min each), secondary antibodies coupled to Cy3 or FITC were applied for 30 min at RT. Then the sections were washed three times with PBS (15 min each), rinsed in water, dehydrated in ethanol, and air-dried. Mounting medium was applied and preparations were preserved by covering them with coverslips and sealing them with nail polish. Immunohistofluorescence was documented with a Zeiss Axioskop epifluorescence microscope (Zeiss, Germany) or with a Leica DM IRE2 (Leica, Wetzlar, Germany).

2.3.7 Expression vectors with *Ptpns1* or *Cd47*

The cDNA sequences containing the complete coding region of mouse *Ptpns1* and *Cd47* were amplified from the reverse-transcribed total RNA used for the semi-quantitative RT-PCRs (see section 2.3.3). To minimize the risk of polymerase-induced mutations, the 'Expand High Fidelity Plus PCR System' (Roche) was used according to the manufacturer's instructions. The annealing temperature of the PCR reactions was 60°C. In addition to the gene-specific sequences, the primers contained restriction sites for *Bam*HI and *Xba*I for efficient cloning of the cDNAs.

PCR reaction for amplification of *Cd47* and *Ptpns1* cDNAs

Reaction mix		Thermal profile	Temperature	Time	Cycle number
Reaction buffer	1x	Initial denaturation	94°C	2 min	1x
dNTPs	200 µM each				
Template cDNA	0.5 µg	Denaturation	94°C	30 sec	
Forward primer	1 µM	Annealing	60°C	30 sec	35x
Reverse primer	1 µM	Elongation	72°C	2 min	
Expand HiFi enzyme blend	1.5 U				
Water	ad. 25 µl	Final elongation	72°C	7 min	1x

Primers for amplification and sequencing of *Ptpns1* and *Cd47*. Restriction sites are underlined, start codons are bold.

Cd47-forward	CGGGATCCGAG**ATG**TGGCCCTTGGCG
Cd47-reverse	GCCTCTAGACCTATTCCTAGGAGGTTGGAT
Ptpns1-forward	CGGGATCCATT**ATG**GAGCCCGCCGGCCC
Ptpns1-reverse	GCCTCTAGACTTCCTCTGGACCTGGACAC
Ptpns1-sequencing1	AAGGTCATCTGCGAGGTAGC
Ptpns1-sequencing2	GATCAAGGGAGCATGCAAAC

The PCR products were purified by gel extraction using the QIAquick gel extraction kit (Qiagen) and their quality was controlled by agarose gel electrophoresis. These sequences were ligated into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) to ensure efficient cloning of the PCR products. 'One Shot competent *E. coli* cells' (Invitrogen) were transformed with the ligation reactions according to manufacturer's instructions and incubated at 37°C overnight on selective plates containing 50 µg/ml ampicillin. Bacterial colonies were further propagated as liquid cultures in Luria Bertani medium containing 10 g/l tryptone (Difco), 5 g/l yeast extract (Difco), 10 g/l NaCl, and 50 µg/ml ampicillin overnight at 37°C, and plasmid DNA was prepared by alkaline lysis with SDS according to Sambrock and Russell (2000). Isolated DNA was digested with the restriction enzymes *Bam*HI and *Xba*I (Fermentas) to identify plasmids containing the respective cDNA inserts. Bacterial clones with proper inserts were further cultivated and kept as glycerol stocks. For production of plasmid vectors, bacteria were grown in Luria Bertani medium overnight at 37°C and harvested by centrifugation (6,000 g,

15 min, 4°C). Plasmid DNA for cloning was prepared with the Qiagen plasmid midi or maxi kit according to the manufacturer's instructions (Qiagen). The expression vector pcDNA4/V5-His A (Invitrogen) was kept as an *E. coli* glycerol stock.

Next, the cDNA inserts were cloned into the expression vector. The pCR2.1-TOPO vector containing the cDNA inserts and the expression vector pcDNA4/V5-His A were digested with *Bam*HI and *Xba*I and purified by gel extraction (Qiagen). Complete digestion was verified by agarose gel electrophoresis and the concentration was measured spectrophotometrically. The insert and vector DNAs were pooled in molar ratios between 1:1 to 1:10 and ligated by incubation with T4 DNA ligase and ligase buffer (Roche) overnight at 15°C. Competent *E. coli* were transformed with the complete ligation reactions and grown overnight on bacterial dishes with medium containing 50 µg/ml ampicillin. Resistant clones were further propagated in liquid culture and screened by digestion with *Bam*HI and *Xba*I, as described above. Those bacterial clones containing a vector with a pcDNA4/V5-His A backbone and a *Ptpns1* or *Cd47* insert (Fig. 10) were selected and the inserts were sequenced using the BigDye Terminator Chemistry (PE Biosystems) with the primers used for cloning and then separated on an ABI 377 DNA sequencer. For the *Ptpns1* cDNA, additional internal primers were required due to the long cDNA sequence. Those clones with a correct sequence were kept as glycerol stocks and used to generate NIH/3T3 cell lines stably expressing *Ptpns1* or *Cd47* (see section 2.1.5).

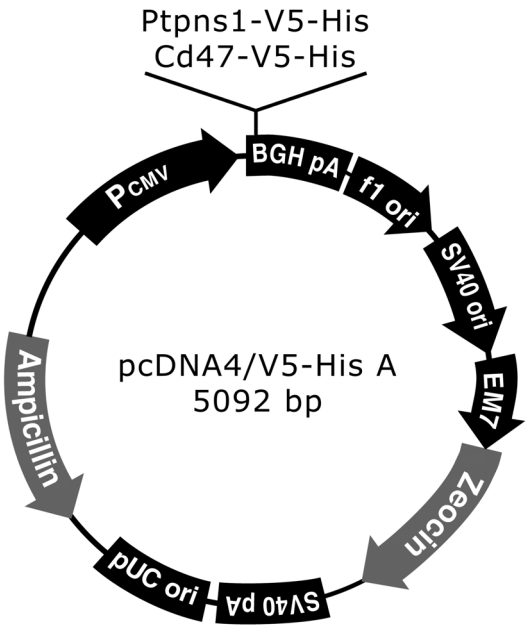

Figure 10. *Ptpns1* or *Cd47* cDNA sequences were inserted into the expression vector pcDNA4/V5-His A (Invitrogen). The cDNAs were fused in frame to a sequence encoding for a V5-tag and a His-tag. The transgene was under control of the constitutively active promoter P_{CMV}. A BGH polyadenylation site behind the transgene ensured stability of the transcript. The resulting vectors were 6566 bp (*Ptpns1*) and 6011 bp (*Cd47*) in size. Genes encoding for proteins that confer resistance to the antibiotics ampicillin and zeocin were present to allow selection. Origins of replication for bacterial and mammalian cells were present. The identity of the vector was verified with selective restriction digests. The insertion of the transgene was confirmed by sequencing.

2.3.8 Immunoblotting

To verify the expression of *Ptpns1* and *Cd47* by the NIH/3T3 cell lines, protein samples were analyzed by immunoblotting. Cell were collected from single 6-wells, resuspended in 50 µl of a modified white Lämmli solution, and boiled for 5 min to yield total cell lysates. The protein concentration was determined using the bicichoninic acid (BCA) assay. To do so, 1 µl of protein extract was diluted in 19 µl of water, mixed with 400 µl BCA solution, and incubated for 30 min at 37°C. The absorption was measured spectrophotometrically at a wave length of 562 nm. 20 µl of water served as reference. A standard curve was established with bovine serum albumin solution of known concentration.

Modified white Lämmli		BCA solution	
Tris	25 mM	Bicinchoninic acid solution	50 vol.
Glycine	192 mM	$CuSO_4 \cdot 5H_2O$ 4% (w/v)	1 vol.
SDS	10%		
Complete mini protease inhibitor cocktail (Roche)	1 tablet/10 ml	(all from Sigma except when stated)	

40 µg of total protein extract in white Lämmli were mixed with an equal volume of 2x blue Lämmli loading buffer, boiled at 98°C for 5 min, and separated on 6% or 10% SDS-PAGE gels using the Mini-PROTEAN 3 electrophoresis system (Bio-Rad Laboratories, Munich, Germany). The separation was done in Lämmli running buffer at 200 V for 45-60 min. The blotting membrane (PVDF, Roche) was equilibrated in methanol (5 sec) and water (2 min). Then the gel and the blotting membrane were gently shaken in blotting solution for 30 min. The proteins were blotted onto the membrane in a Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad) at 15 V for 30 min. To saturate the unbound regions on the blot, it was incubated in TBST/5% milk powder for 1 h at RT or overnight at 4°C. Then it was incubated with the primary antibody diluted in TBST/1% BSA for 1 h at RT or overnight at 4°C followed by three washing steps with TBST. The secondary antibody was diluted in TBST/1% BSA and added to the blot for 30 min. After another three washing steps in TBST, the signals were visualized with the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Rodgau-Jügesheim, Germany) and a Fuji Medical X-Ray Film (Fuji, Düsseldorf, Germany) in a Curix 60 automatic film processor (AGFA, Cologne, Germany).

SDS-PAGE separating gel		SDS-PAGE stacking gel	
Tris-HCl, pH 8.8	375 mM	Tris-HCl, pH 6.8	125 mM
Acrylamide	6% or 10%	Acrylamide	4%
SDS	1%	SDS	1%
Ammonium persulfate (APS) (10%)	0.005 vol.	Ammonium persulfate (APS) (10%)	0.008 vol.
TEMED	0.001 vol.	TEMED	0.002 vol.

Lämmli running buffer		2x blue Lämmli loading buffer	
Tris-Base	3 g/l	Tris-HCl, pH 6.8	125 mM
Glycine	14 g/l	SDS	4%
SDS	0.1%	Glycerol	20%
		DTT	200 mM
		Bromphenolblue	0.04%

Blotting solution		TBST	
Tris-Base	5.82 g/l	Tris-Base	10 mM
Glycine	2.93 g/l	NaCl	150 mM
SDS	0.38%	adjust pH to 8.0	
Methanol	20%	Tween-20	0.1%
Bromphenolblue	0.04%		

Antibodies	Supplier	Dilution
anti-PTPNS1 (Sirpα)	BD Biosciences	1:100
anti-CD47 (IAP, miap301)	BD Biosciences	1:100
anti-Actin	Sigma	1:500
anti-V5	Invitrogen	1:800
anti-mouse-HRP	Dianova	1:2,000
anti-rabbit-HRP	Amersham	1:2,000
anti-rat-HRP	Santa Cruz	1:1,000

2.3.9 Culture of neural progenitors on fibroblasts and in conditioned medium

To test whether neurosphere cells migrated or differentiated differently depending on the presence or absence of PTPNS1 or CD47, NIH/3T3 cells stably expressing the respective transgenes were plated onto coverslips and reached confluency after 2 days. Then neurospheres were resuspended in D-MEM/F12 supplemented with B27, 5% fetal bovine serum, and HEPES buffer, and plated onto the NIH/3T3 monolayer. After 4 days, the cells on coverslips were fixed with methanol and acetone, and analyzed by immunofluorescence (see section 2.3.5).

To test whether neurosphere cells migrated or differentiated differently depending on the conditioned medium, more specifically on the presence or absence of potentially shedded ectodomains, medium from NIH/3T3 fibroblasts and stable cell lines was removed from these cultures after 2 days and sterile-filtered. Neurospheres were resuspended in these conditioned media and plated onto coverslips covered with poly-L-lysine. 6 days after induction of differentiation the cells on coverslips were fixed with methanol and acetone, and analyzed by

immunofluorescence (see section 2.3.5). The number of Ki-67 positive cells per neurosphere was counted. Arithmetic mean and standard deviation were determined with Microsoft Excel. The Mann-Whitney Test (U-Test) was performed with SPSS 9.0 (SPSS Inc., Chicago, IL, USA).

2.3.10 Bioinformatics and database analyses

Many different bioinformatics tools and databases were used during this study. Tasks included the analysis of sequencing data for the verification of clones on the array, mapping clones in the mouse genome, and designing primers for the semi-quantitative RT-PCRs and for the cloning of *Ptpns1* and *Cd47*. Concerning the candidate genes showing expression changes in the course of neural progenitor cell differentiation, extensive literature searches were performed. In addition, the expression and splicing of these candidate genes, and the corresponding proteins were studied. The subcellular localizations of these proteins were predicted. Tools and databases used for these studies, and their world wide web addresses are listed below.

National Center for Biotechnology Information, Bethesda, MD, USA
PubMed, UniGene, Nucleotide, Blast Search (http://www.ncbi.nlm.nih.gov/)

Genome Bioinformatics Group, University of California, Santa Cruz, CA, USA
Genome Browser, Blat Search (http://genome.ucsc.edu/)

European Bioinformatics Institute, Hinxton, UK and The Wellcome Trust Sanger Institute, Hinxton, UK
Ensembl (http://www.ensembl.org/)

The Wellcome Trust Sanger Institute, Hinxton, UK
Protein families database of alignments and HMMs (Pfam)
(http://www.sanger.ac.uk/Software/Pfam/)

Swiss Institute for Bioinformatics, Switzerland
Swiss-Prot - Protein Knowledgebase (http://www.expasy.org/sprot/)

Weizmann Institute of Science, Rehovot, Israel
GeneCards (http://bioinformatics.weizmann.ac.il/cards/)