3 RESULTS

3.1 Neural progenitor cells *in vitro*

3.1.1 Establishment of the neural progenitor cell culture

To establish neural progenitor cell cultures, several protocols from previous studies were compared (Doetsch et al., 1999; Svendsen et al., 1998; Geschwind et al., 2001; Laywell et al., 2002). These usually had been optimized for the respective cells of interest, e.g. embryonic neural precursors or neural precursors from the dentate gyrus. A first goal was to cultivate subventricular zone progenitor cells with defined medium components only and without serum to facilitate the comparison of different cultures. Several factors were optimized, e.g. the initial procedure of cell isolation from the brain, the culture composition, and the timing of medium change and replating. Since the cells grow in suspension as free floating balls, called neurospheres, it is necessary to break these spheres apart for efficient replating of single cell suspensions. This dissociation of neurosphere cells can be done by protease digestion followed by mechanical separation and turned out to be a critical step. Initial experiments with papain or trypsin resulted in poor separation of the neurosphere cells or reduced their viability. In addition, the inactivation of these enzymes after their application is usually done by serum. Serum, however, was to be avoided. Optimal digestion results were achieved by treating the cells with a commercially available mix of the self-inactivating protease accutase, collagenase, and DNase, and then carefully separating them with a fire-polished pasteur pipette.

Neurospheres appeared after about five to six days after dissection (Fig. 11). After ten to twelve days neurosphere cells were separated by digestion and replated. Centrifugation at low speed allowed to separate neurospheres from other cells and cellular debris in the culture and to selectively replate these cells. From these single cells, secondary neurospheres arose within eight to ten days and could be replated likewise. While the primary preparation was always contaminated with cellular debris from the surrounding tissue, the following passages were pure and clean, since only the neural progenitors proliferated under the rather specific media conditions.

3.1.2 Differentiation of neural progenitor cells *in vitro*

Neurosphere cells need to attach to a surface in order to differentiate. Also, EGF keeps the cells in a proliferative state and prevents differentiation (Reynolds and Weiss, 1992). Thus, neurospheres were transferred into dishes and onto coverslips coated with poly-L-lysine and removed the EGF from the medium. To support neuronal survival and maturation, either neurotrophin-4 (NT4) or brain-derived neurotrophic factor (BDNF) was added to the differentiating cells

(Caldwell et al., 2001). The cells attached within a few hours. Single cells started to migrate out of the spheres. Over time, more and more cells migrated away from the sphere (Fig. 11, 24 h -EGF, 96 h -EGF). After eight to twelve days, the whole cluster disappeared. The cells changed their morphology and appearance. Some acquired a flat, large, and dark appearance typical for glial cell (Fig. 11, arrow a). Others reduced their cytoplasmic volume, extended long slim processes which sometimes connected to other cells, and had a brightly shining cell body (Fig. 11, arrow b). Other cells developed a complex net-like system of processes (Fig. 11, arrow c). The latter two morphologies are typical for neuronal cells.

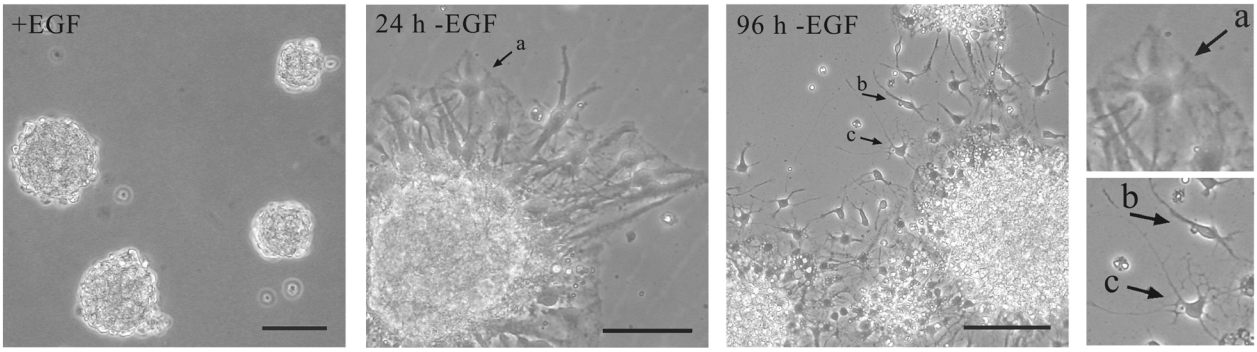


Figure 11. Neural progenitor cell growth and differentiation *in vitro*. Neural progenitors form large aggregates called neurospheres when proliferating *in vitro* (+EGF). After induction of differentiation, cells migrate out of the spheres and acquire different morphologies (24 h -EGF, 96 h -EGF, arrows a, b, c). Scale bar equals 50 µm.

Immunofluorescence analysis was used to characterize the cells before and after induction of differentiation. Glial fibrillary acidic protein (GFAP) is a marker protein that specifically labels astrocytes (Dahl and Bignami, 1973; Bignami and Dahl, 1974). Young neurons are frequently detected using the monoclonal antibody Tuj1, which recognizes tubulin-β-III, a neuron-specific tubulin isoform (Lee at al., 1990). The neurosphere cells were GFAP-positive during proliferation. After induction of differentiation and migration, cells in close proximity to the sphere appeared as large flat glia-like cells, and they were still positive for the astrocyte marker GFAP (Fig. 12, upper panel). In contrast, cells that migrated further away from the sphere acquired a neuronal morphology with small cell bodies and long, slim processes. These were positive for tubulin-β-III (Fig. 12, upper panel). Most cells within the spheres were positive for GFAP, however, after 96 h the compact sphere structure had frequently loosened up and some of these cells had changed their morphology as well. A few cells inside the spheres produced tubulin-β-III after induction of differentiation, whereas most differentiating cells were GFAP-positive. A very small proportion of cells was positive for the oligodendrocyte marker GALC (not shown).

To test for proliferation, cells were immunostained with an antibody against Ki-67, a

marker for dividing cells (Gerdes et al., 1983). Before induction of differentiation, some cells within the neurospheres were positive for Ki-67. Twenty-four hours after induction of differentiation, however, no more dividing cells were found (Fig. 12, middle panel). The cells were also immunostained for nestin, a neuroepithelial precursor cell marker (Lendahl et al., 1990). Before and after induction of differentiation, neurosphere clusters were nestin-positive. Cells that had migrated away from the spheres, however, no longer expressed nestin (Fig. 12, bottom panel).

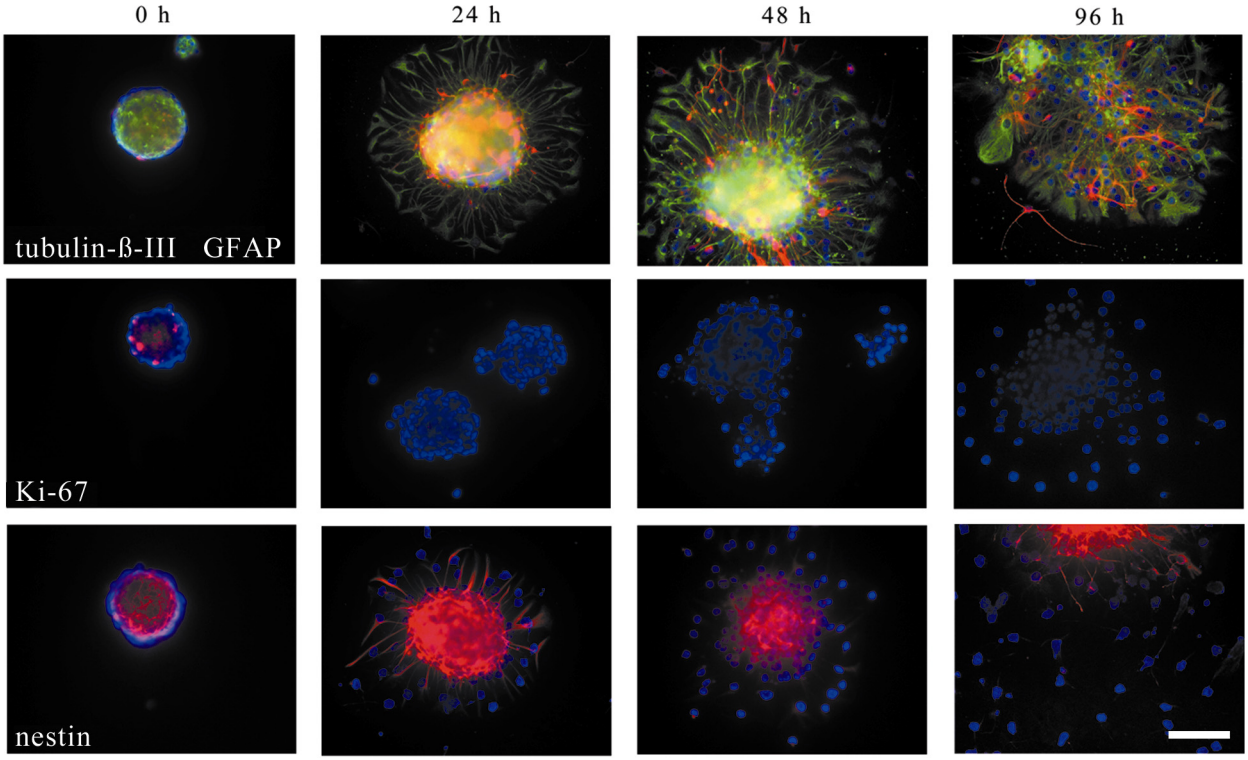


Figure 12. Immunofluorescence microscopy of undifferentiated neurospheres and of cells at 24 h, 48 h, and 96 h after induction of differentiation. Cells were immunolabeled with antibodies against tubulin-β-III, Ki-67, nestin (all red), and GFAP (green). Nuclei were stained with DAPI (blue). Note the different staining of undifferentiated neurosphere clusters and differentiating cells. Scale bar equals 100 µm.

3.1.3 Monolayer progenitor cell cultures

Neural progenitors can not only be grown as neurospheres but also as monolayer cultures. This offers some advantages concerning the handling of the cells, since the separation of the sphere clusters would be dispensable. Therefore, a modified protocol adapted from an experimental procedure by Palmer et al. was used (1995). Briefly, the cells were isolated as before, but plated onto dishes coated with a mixture of poly-L-ornithine and laminin. In addition, for the first day *in vitro* the cells were not exposed to EGF or bFGF but were incubated with fetal bovine serum. Under these conditions all cells attached to the surface. After one day the medium supplement was switched from serum to bFGF and EGF. After another four days bFGF was also removed.

This procedure caused progenitor cells to proliferate, whereas other cells did not. Passaging of the cells resulted in an increased morphological homogeneity of the culture. These cells were long and slim and sometimes reminiscent of fibroblasts (Fig. 13 a, b). Many of these cells were undergoing mitosis, as was detected by immunostaining using the proliferation marker Ki-67 (Fig. 13 c). In addition, they were positive for the precursor marker nestin (Fig. 13 c). Staining for tubulin-β-III revealed that only a few cells expressed this early neuronal marker. It was already present, however, in a number of cells growing in EGF-containing medium (Fig. 13 d). Most of the cells were positive for GFAP (Fig. 13 d).

To induce differentiation, these monolayer neural progenitor cells were deprived of EGF. The morphology of many cells changed significantly. They developed additional extensions reminiscent of neural cells. Other cells increased in cell body size (Fig. 13 e, f). Characterization by immunofluorescence showed that Ki-67 was no longer detectable (Fig. 13 g). Signals corresponding to the nestin protein were weaker, though still detectable (Fig. 13). This indicates that EGF removal caused a proliferation stop. Tubulin-β-III signals were much stronger compared to proliferating cells (Fig. 13 h). GFAP was detected at an intensity comparable to the proliferating culture (Fig. 13 h).

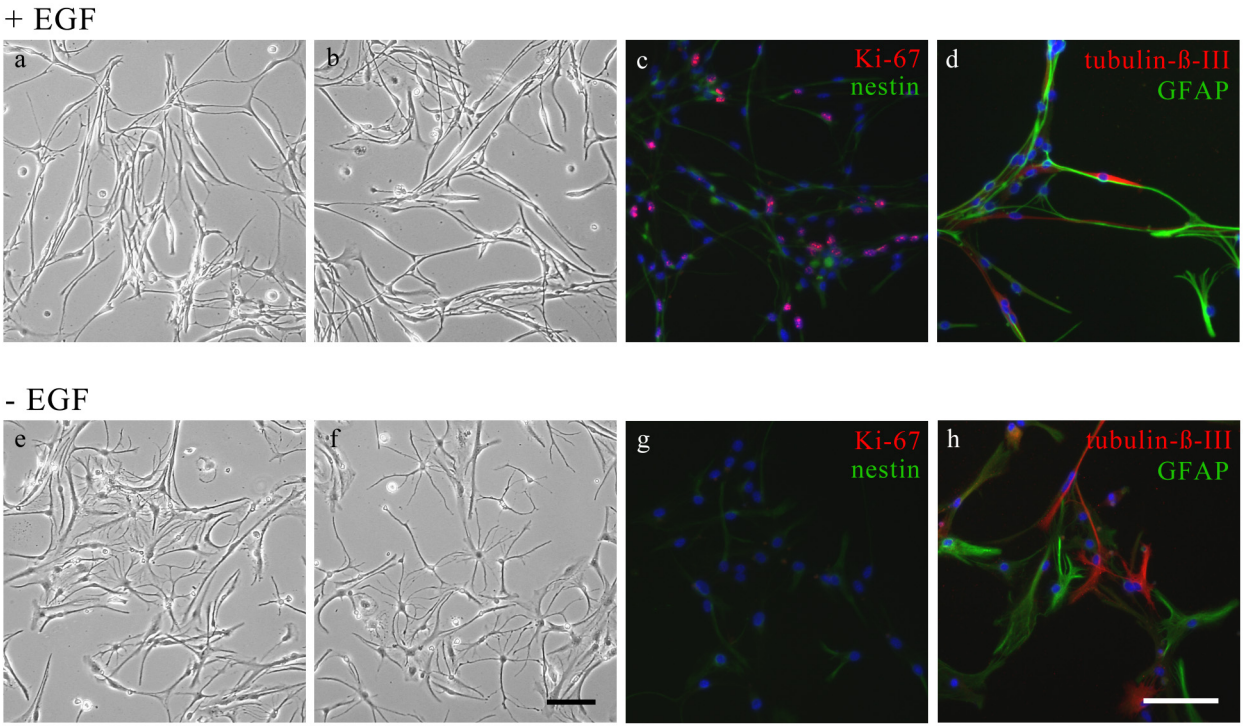


Figure 13. Neural progenitor cells kept as monolayers in medium supporting proliferation (+ EGF) or differentiation (- EGF). Depicted are photographs showing the morphology of cells in a, b, e, and f, as well as immunofluorescence analysis of cells during proliferation (c, d) and after induction of differentiation (g, h). These cells were immunolabeled with antibodies against Ki-67 or tubulin-β-III (red), and nestin or GFAP (green). DNA staining was performed with DAPI to label the nuclei (blue). Scale bar equals 50 µm.

3.2 Expression profiling of neural progenitor cells

3.2.1 Microarray analysis

To identify genes involved in neural progenitor cell differentiation, microarray experiments were performed. The microarrays were produced in our lab as described in section 2.2.1. They contained 13,627 clones.

The neurosphere cells used for the microarray analyses had been taken into culture at postnatal day seven and were kept *in vitro* for 27 days. During this time, the neurospheres had been replated twice by dissociation and dilution. Then, differentiation was induced by removal of EGF, addition of other growth factors to stimulate differentiation, and plating into dishes and onto coverslips coated with poly-L-lysine. Two experimental series were performed, one supplied with brain-derived neurotrophic factor (BDNF) and one with neurotrophin-4 (NT4). In each series, cells for RNA preparation and cells grown on coverslips were collected from proliferating neurosphere cells and from differentiating cells after 24 h, 48 h, and 96 h. The coverslips were used for immunofluorescence analyses of relevant marker proteins and potential candidate gene products. Photographs showing the cells at relevant time points are given in Fig. 14.

The RNA samples were used for hybridizations as shown in Fig. 14. A sample from undifferentiated cells was always co-hybridized with a sample from cells after induction of differentiation. Since I had collected RNA from three time points after induction of differentiation, this resulted in a time course study of gene expression changes over the first four days of differentiation *in vitro*. To compensate for dye-specific effects, every hybridization was repeated with the fluorescent dyes swapped. Therefore, three time points in two experimental series with a dye swap for every hybridization resulted in twelve co-hybridizations in total.

The twelve co-hybridizations, six per series, generated twelve single datasets that were subjected to further analysis. This involved a normalization step (see section 2.2.3), resulting in three datasets per series, one for each co-hybridization (0 h versus 24 h, 48 h, or 96 h). In other words, for every clone on the array, six data points were obtained corresponding to the expression change relative to the undifferentiated state at days one, two, and four in the BDNF series or the NT4 series. To identify differentially expressed genes, an estimated two-fold difference in expression was considered relevant. This threshold is based on a statistical analysis as described in section 2.2.3. It revealed that when considering all clones above a two-fold change, two to five percent of the signals are false positives.

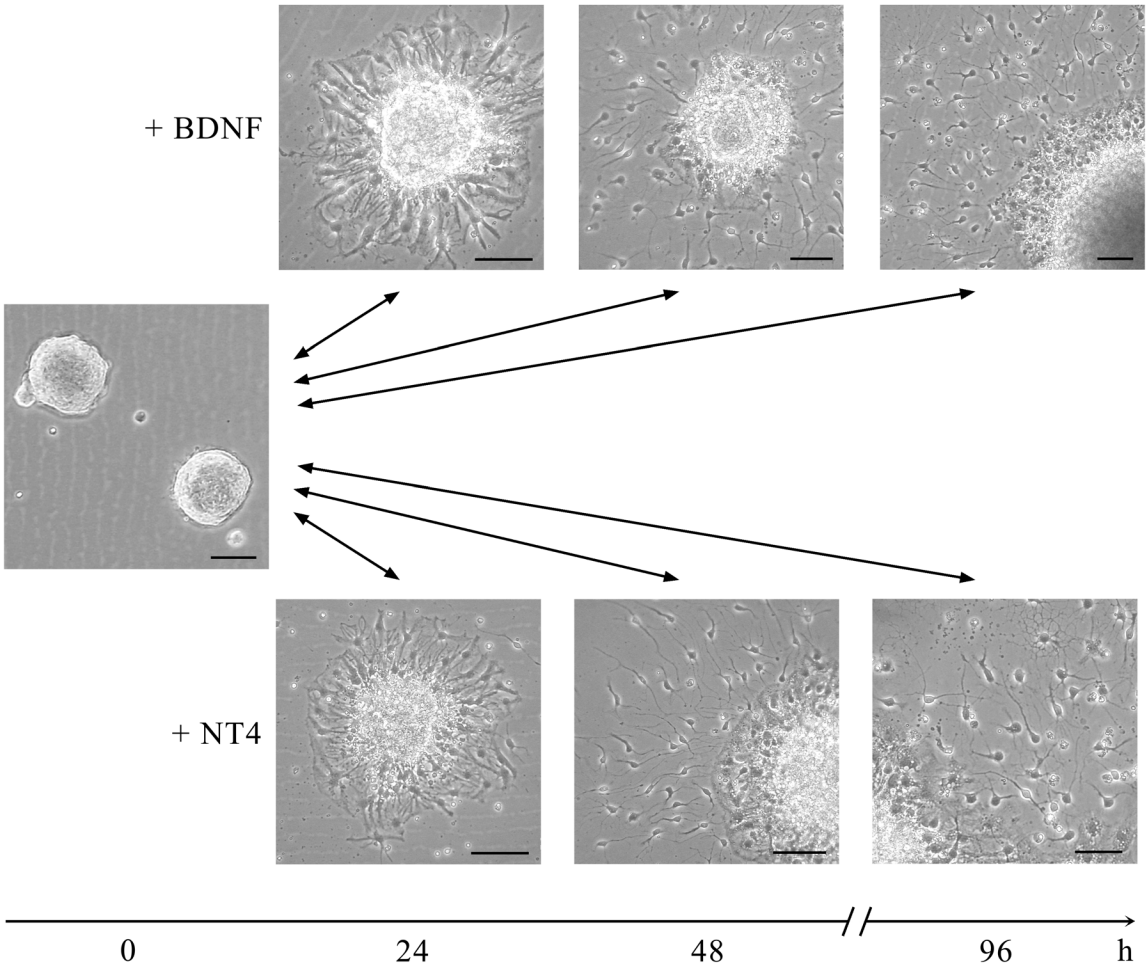


Figure 14. Neural progenitor cell differentiation *in vitro*. The photographs depict morphological changes associated with adhesion, migration, and differentiation of neural progenitor cells within the first four days after induction of differentiation. Either neurotrophic growth factor BDNF or NT4 was added. Arrows indicate the microarray hybridization scheme, where RNA from differentiated cells and, as a reference, RNA from undifferentiated cells were used for co-hybridizations. Scale bars equal 50 µm.

To be selected for further analysis, a clone had to exhibit a more than two-fold difference in expression in at least one of the three time points (day one, two, or four). 722 clones of the BDNF experiment and 624 clones of the NT4 experiment met this criterion. To find common biological effects related to neural differentiation, the intersection of both lists was taken. These were 454 clones. In addition, when considering only the intersection of both series, the likelihood of false positives is strongly reduced. Therefore, further analyses concentrated on the 454 clones of the intersection list, thus focusing only on the commonalities associated with the TRKB receptor activation in the two experimental series. Thus, 63% of the 722 BDNF clones were found in the NT4 list, and 73% of the 624 NT4 clones were found in the BDNF list of differentially expressed genes. Of the 722 clones from the BDNF series, 83% showed at least a 1.6-fold estimated change in the NT4 experiment, and 84% of the 624 clones from the NT4

series showed at least a 1.6-fold estimated change in the BDNF experiment. The enormous overlap of results from these two independent experiments suggests that the neurotrophins BDNF and NT4, both of which bind to the TRKB receptor, lead to largely overlapping gene expression changes. Nonetheless, the remaining clones specific to either the BDNF or the NT4 list were evaluated as well (see below).

Of the 454 clones, 97.1% were consistently up- (271 clones) or down-regulated (170 clones) at the three time-points (negative or positive log ratio of signal intensity) compared to undifferentiated cells in both series. 2.9% (13 clones) showed an up-regulation at one time-point and subsequent down-regulation or vice versa (e.g. *phosphoglucose isomerase 1*, see Table 1, metabolism, NT4 series).

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, which showed comparable expression changes, it was not sequence-verified.

The complete dataset (rendered using MIAMI standards) including remaining clones with relevant expression changes, which are not listed in these groups, can be found on the lab homepage (http://www.molgen.mpg.de/~dna_microarrays/neural_differentiation/neural.html).

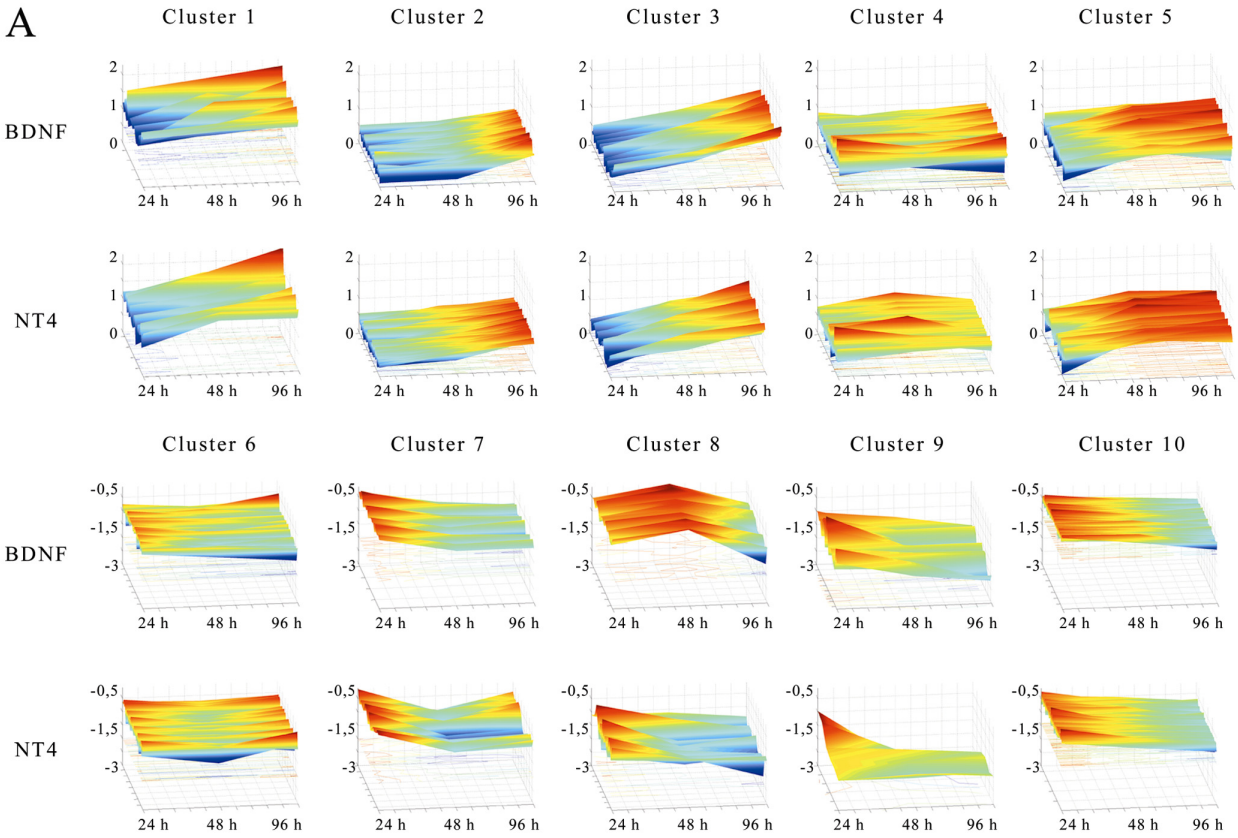
3.2.2 Cluster analysis

To extract the dynamic behavior of the relevant gene expression changes in the course of neural progenitor differentiation, a cluster analysis was performed using the 441 consistently up- or down-regulated clones from the intersection list (see section 2.2.4). The 441 clones were grouped into ten clusters which showed a similar onset and course of transcriptional changes (Fig. 15 A). In both the BDNF and the NT4 series, clusters one to five contained up-regulated clones and clusters six to ten down-regulated clones. With the exception of clusters seven and eight, there was a large fraction of clones in each cluster that occurred in both series (Fig. 15 B, Table 1). Most genes fell into clusters that displayed only slight expression changes (clusters two, four, five, six, seven, and ten). Clusters one and three contained strongly up-regulated genes, and clusters eight and nine contained strongly down-regulated genes.

Concerning the dynamics, genes that changed late in expression were especially found in cluster two. Genes that changed very early (between time point 0 h and 24 h) were found in two of the four clusters with strongest changes (clusters one and nine). In addition, they were found in cluster six. Down-regulated genes of the functional category ‘chromatin-associated components and nuclear factors’, fell into clusters six and ten, which contained slightly decreasing transcript levels. Cluster nine included numerous cell cycle-related genes. Cluster two

(late up-regulated transcripts) contained many genes whose products localize to the cell membrane and/or are found at cellular extensions. Apart from these findings, single clusters did not preferentially contain genes of a certain category.

A



B

Cluster	1	2	3	4	5	6	7	8	9	10	Sum
BDNF	24	51	36	106	54	39	21	15	18	77	441
NT4	22	82	30	74	63	39	16	18	6	91	441
Intersection	21	35	12	55	33	22	3	1	6	61	249

Figure 15. (A) Clusters of clones with similar expression changes over time. The dynamic behavior of the 441 clones that were consistently up- or down-regulated in both, the BDNF and the NT4 series, is shown separately. For each series ten clusters are shown. Clusters one to five represent up-regulated, clusters six to ten down-regulated clones. The x-axis depicts the three time points of differentiation. The y-axis shows relative fold changes, that is expression changes referred to the undifferentiated state (0 h). These relative numbers are estimated logarithmic fold changes and therefore not identical to the estimated fold changes listed in Table 1. The logarithmic values allow a better graphical presentation. Note that y-axis values at 24 h that are different from the value at 0 h indicate that the expression of a clone changed already between 0 h and 24 h. (B) Numbers of clones contained in each cluster of the NT4 and BDNF series.

3.2.3 Literature and database analyses

To collect meaningful biological information about the differentially expressed genes, an extensive literature survey was conducted. Relevant publications describing the structure or function of genes that were found in this study were screened for valuable information. In addition, the domain structures of the encoded proteins were analyzed to find motifs related to the localization or function of the respective protein. This information allowed to assign many of the clones to categories according to their localization or function. Of the 454 clones that exhibited a differential regulation in the experiment, 161 corresponding genes which might be interesting to look at in more detail in the context of neural progenitor cell differentiation were grouped into ten categories. These categories and the respective genes are listed in Table 1. For instance, detachment from the neurosphere cluster, migration, and development of cellular processes such as dendrites and axons involves genes of the categories 'cell-cell adhesion' & 'cytoskeleton'. Differentiating cells stop proliferation, and genes related to this event are in the 'cell proliferation' category. The transition from an immature cell type to a differentiated one involves several signal transduction pathways and is regulated by transcription factors. Genes that might be involved in this process are in the corresponding categories 'signal transduction' and 'chromatin-associated components and nuclear factors.'

Previously, large scale gene expression analyses have only been used to study undifferentiated neurosphere cells or to compare these to only one cellular state after induction of differentiation. Geschwind et al. (2001) investigated undifferentiated neurosphere cells from murine prenatal day one striatum and cells differentiated for 24 h using a combination of representational difference analysis and microarrays. Karsten et al. (2003) compared similar undifferentiated cells and cells that were differentiated for 24 h. Easterday et al. (2003) compared postnatal day one cortical neuropheres differentiated for 24 h to three different undifferentiated neurosphere populations from mice. Cells derived from the striatum of rat embryos were used in two other differentiation studies by Zhou et al. (2001) and Wen et al. (2002). These findings were compared with the results of this study. Of the 161 genes listed in Table 1, 66 had been detected in neurosphere cells by these microarray studies (Tab. 1). In most cases the microarrays used in these studies contained fewer features than ours, thereby limiting the number of genes that can be detected. Wen et al. even hybridized RNA from rat neurospheres onto a cDNA array with human sequences. In addition, performing a time course and including later time points in the analysis detected many more differentially expressed genes. This is supported by the notion that for most clones the fold change in expression increased over time. Genes that had been mentioned in these previous studies are marked in Table 1. This represents an independant confirmation of the results obtained in this study.

Gene expression changes in neural progenitor cells during differentiation

Gene	Trend	Cluster	Fold Change (BDNF) 24 h	48 h	96 h	Cluster	Fold Change (NT4) 24 h	48 h	96 h	Accession number	mentioned previously
Cell-cell-adhesion											
Cadherin 13	-	7	1,35	2,97	3,10	7	1,12	3,03	3,42	NM_019707	1, 5, 6
EST adjacent to cadherin 13	-	9	1,52	4,90	6,69	8	1,45	4,48	5,87	AW121058	1, 5, 6
Cd24	+	2	1,57	1,73	2,23	2	1,49	1,82	2,39	AW553321	4, 6
Close homologue of neural Chl1	-	6	1,88	2,36	2,36	7	1,65	2,77	2,80	AI528777	5
Epithelial membrane protein 3	+	2	1,31	1,14	2,39	2	1,12	1,26	2,05	AW912730	
Neurexin II	+	4	1,77	1,86	2,03	4	1,72	2,03	1,99	BE286506	
Plakophilin-4 (p0071 protein)	+	5	1,84	3,00	3,35	5	1,58	3,10	3,49	AI837226	
Extracellular matrix components and cell-matrix interaction											
Adamts10	+	2	1,52	1,70	2,44	2	1,60	1,72	2,01	BG064835	
AE binding protein 1	+	2	1,25	1,16	2,97	4	1,58	2,27	2,03	BF118240	
Collagen IX alpha 3	+	4	2,16	2,12	1,72	4	2,08	1,82	1,60	AV006866	5
Collagen XVIII alpha 1	-	10	1,36	1,68	2,51	10	1,38	1,68	2,01	U03714	6
Complement component 1 q beta	+	4	2,29	2,48	2,75	5	2,01	2,56	3,19	AW208835	
Complement component 3	+	1	2,27	7,24	7,61	1	1,55	4,31	6,89	AI790152	
Complement component 4	+	1	4,10	5,47	6,17	1	3,60	6,23	6,17	BE369103	
Complement component factor i	+	3	1,42	2,18	3,32	3	1,07	2,44	3,06	BG072327	
Extracellular matrix protein 2 (Sparc-like 1)	+	5	2,25	3,19	3,49	5	1,63	3,06	3,56	W36427	
Inter-alpha trypsin inhibitor heavy chain 3	+	3	2,12	2,46	4,31	3	1,82	3,29	4,06	NM_008407	
Laminin receptor 1	-	10	1,31	1,67	2,03	10	1,57	1,63	2,39	W13846	6
Serpina3	+	2	1,39	1,39	2,29	4	2,03	1,55	1,68	BE200266	
SH3 domain protein 4 (Vinexin / SCAM-1)	+	3	1,03	1,70	3,39	2	1,17	1,75	2,18	AW540876	
Tenascin C	-	7	1,62	2,53	2,36	6	2,12	2,25	1,72	AI157473	2, 4, 5, 6
Tissue inhibitor of metalloproteinase 2	+	4	2,20	2,41	3,16	5	1,79	2,72	2,92	W41950	5
Tissue inhibitor of metalloproteinase 3	+	4	2,44	1,88	1,95	4	1,80	2,01	2,14	AA712082	2
Cytoskeleton											
Actinin, alpha 1 (Striamin)	+	5	2,18	2,89	2,92	5	1,67	2,59	2,48	BE688759	
Active BCR-related gene	+	5	1,72	3,10	3,16	5	1,75	3,03	2,72	BF449832	
Adducin 3	+	4	1,75	1,97	2,18	4	1,82	2,23	2,18	AA500258	
Arhgap18	-	6	2,20	2,86	2,53	10	1,90	2,20	2,77	BG071822	
Arhgap5	+	4	1,49	2,03	1,48	2	1,23	1,38	2,03	NM_009706	5
Cdc42 effector protein 4	+	3	1,42	2,48	3,16	3	1,48	2,16	2,94	AI844806	6
N-chimerin	-	6	1,97	2,80	2,86	10	2,18	2,36	3,29	BG088668	
Coronin actin binding protein 1C	-	10	1,40	1,99	2,08	10	1,46	1,93	2,18	AW455561	
Cysteine-rich protein 1	+	4	1,75	2,39	2,29	4	2,08	2,12	2,18	W18400	5
Dynein, axonemal, heavy polypeptide 7	+	4	3,00	1,79	2,80	4	2,53	3,42	1,86	BG292586	
Dynein, cytoplasmic, light chain 2B	+	2	1,54	1,68	2,05	2	1,52	1,31	2,16	BG083926	
Ezrin (villin 2)	+	4	2,25	1,93	2,44	2	1,82	2,05	2,53	AA139292	6
Gelsolin	+	1	3,00	4,71	6,05	1	2,69	4,35	6,11	AI848142	
Kinesin heavy chain member 5B	+	4	1,70	2,10	2,16	2	1,40	1,46	2,29	AI892690	4
Leucine rich repeat (in FLII) interacting prot. 1	-	8	1,86	1,23	2,92	10	1,40	2,10	3,06	AI461880	2, 4, 6
Neuronal protein 4.1	+	2	1,60	1,70	2,16	2	1,32	1,80	2,41	AW049949	
Plexin B3	+	4	1,31	2,05	1,99	4	1,51	2,41	2,16	BE136019	
Rac GTPase-activating protein 1	-	10	1,70	2,05	1,82	10	1,80	1,92	2,10	AB030252	1, 2, 5, 6
S100 calcium binding protein A1	+	4	1,49	1,95	2,36	2	1,27	1,73	2,36	AK012578	2
S100 calcium-binding protein A4	+	5	1,88	2,41	2,72	5	1,77	2,66	3,32	X05835	
S100 calcium-binding protein A6	+	3	1,84	2,32	3,19	5	1,90	2,36	3,10	AK008241	2
S100 protein beta polypeptide neural	+	5	1,70	2,80	3,74	3	1,22	2,72	3,90	AI835699	
Sperm associated antigen 6	+	2	1,23	1,49	2,64	2	1,19	1,75	2,03	NM_015773	
Thymosin, beta 10	-	6	2,12	2,27	2,61	6	2,29	2,23	2,61	BE135516	5, 6
Transgelin 3	+	3	1,32	2,12	2,59	2	1,30	2,01	2,59	AI426007	
Tropomodulin 2	+	4	1,72	2,20	2,56	5	1,57	2,34	2,41	BB462768	5
Tubulin, alpha 3/7	+	2	1,11	1,49	2,36	2	1,35	1,88	2,59	NM_009446	4
Tubulin, beta 3	+	4	1,86	1,67	2,10	4	1,65	2,29	1,97	AK014302	5, 6
Tubulin, beta 4	+	5	1,34	2,86	2,89	5	1,54	3,06	2,86	AI849897	5
Cell proliferation											
Cdc20	-	9	3,63	6,55	7,10	9	6,69	5,81	6,96	C87100	
Cdc2a	-	9	5,53	4,66	10,07	9	5,93	9,49	8,41	AW990601	1, 2, 4, 6
Chromatin assembly factor 1 subunit C (Rbbp4)	-	10	1,49	1,92	2,23	10	1,42	1,75	2,39	AA139889	
Cyclin B1	-	6	3,71	3,35	3,94	6	3,32	3,97	3,03	AI131574	2, 4
Cyclin D1	-	9	5,37	7,69	6,36	9	5,26	6,23	7,69	BE995549	1, 2, 3, 4, 5, 6
Cyclin D2	-	9	2,25	4,62	4,53	7	1,93	4,62	2,94	NM_009829	1, 2, 3, 5, 7
DNA polymerase delta 1	-	8	2,05	1,40	2,53	6	1,79	2,53	2,36	AI851668	4, 6

DNA polymerase delta subunit 4	-	7	1,38	2,20	2,01	10	1,39	1,73	2,05	AK010477	
DNA replication licensing factor MCM3	-	6	2,92	3,60	4,53	8	3,46	4,01	3,67	BE200058	6
DNA replication licensing factor MCM6	-	10	1,45	1,73	2,01	10	1,32	1,73	2,66	BE854336	2
Fusion 1 (Tusc2)	-	8	1,30	1,45	3,53	7	1,80	3,53	1,82	AA672013	
Kinesin family member 23	-	7	1,73	2,86	3,86	6	3,03	2,94	3,03	AA792884	
PDZ-binding kinase	-	9	5,21	6,69	10,49	9	5,93	7,24	6,62	AW538537	1, 3
Pituitary tumor-transforming protein 1/Securin	-	6	2,77	4,22	2,36	6	3,00	4,10	3,29	AA014315	2, 5
Signal transduction											
CamkII beta	-	9	3,78	4,81	5,10	8	3,16	4,39	6,05	AI849170	5
Calmodulin 1	+	4	1,67	1,95	2,08	4	1,30	2,29	2,05	BG076172	
Calmodulin-dep. calcineurin A subunit alpha	-	10	1,92	1,95	2,44	6	1,58	2,08	1,63	AI528006	
CDP-diacylglycerol synthase 2	+	2	1,30	1,23	2,72	2	1,06	1,31	2,44	AA407759	
Cornichon-like (Cnil)	-	6	2,03	3,06	2,77	10	1,90	2,59	3,03	AI854642	
DARPP-32 (Ppp1r1b)	+	3	1,23	1,67	2,86	3	1,23	1,70	3,60	BF582544	
Deiodinase iodothyronine type II	-	10	1,38	1,77	2,75	10	1,31	1,86	3,22	AF096875	
Deltex 1 homolog (Drosophila)	+	4	1,58	1,90	2,12	4	1,09	2,23	1,46	U38252	5, 6
Endothelial differentiation gene 3	+	4	1,86	2,05	2,80	5	1,72	2,25	2,64	BE950940	
Immediate early response 3 (Ier3, IEX-1)	-	6	2,56	2,44	2,92	6	1,86	2,41	1,79	BF681888	6
Insulin-like growth factor binding protein 2	-	9	1,93	7,85	16,28	9	2,86	9,21	25,79	AI303805	4, 5, 6
Insulin-like growth factor binding protein 4	-	9	2,23	5,58	9,87	8	2,08	4,44	8,85	W08013	
Insulin-like growth factor binding protein 5	+	1	3,29	4,39	4,48	1	3,32	3,60	4,39	W13388	5, 6, 7
Latent transforming growth factor bp 4	+	4	1,43	1,86	2,01	2	1,43	1,58	2,05	AA051293	
Membrane protein, palmitoylated (Emp55)	+	2	1,34	1,42	2,08	2	1,26	1,49	2,20	AI528528	
Neurotensin receptor 2	+	5	2,32	3,49	3,82	5	2,51	3,10	2,92	NM_008747	5
Nuclear receptor subfamily 4 A1 (TR3)	-	6	1,86	2,56	3,32	7	1,58	2,51	2,14	AA118943	
Phosphodiesterase 2A, cGMP-stimulated	-	10	1,42	1,68	2,32	7	1,38	2,25	1,73	BC029810	
Phospholipase A2, group VII	+	4	1,54	1,88	2,12	2	1,34	2,16	2,32	AA183265	4
Phospholipase C delta	+	4	1,90	2,32	2,34	4	2,12	3,00	2,25	AI892312	5
Protein kinase C beta	-	6	2,12	2,80	2,59	6	2,01	2,59	1,75	BF658909	
Ptpns1 (Shps-1)	+	1	2,61	4,76	4,48	1	2,27	3,71	4,39	D87968	
Regulator of G-protein signaling 16	-	6	2,64	2,36	3,82	8	2,97	3,03	3,97	AI876531	
Regulator of G-protein signaling 2	+	2	1,09	1,34	2,66	2	1,17	1,70	2,44	BF100893	
Thyroid hormone receptor interactor 6	+	2	1,39	1,57	2,94	3	1,28	1,90	3,19	AW763698	
Tnf receptor-associated factor 1	+	3	1,20	1,62	3,22	5	1,01	2,72	2,41	BG064103	
Tnf receptor superfamily member 21	-	7	1,32	2,14	2,89	10	1,54	1,48	2,53	AI552031	
Chromatin-associated components and nuclear factors											
1700012H05Rik (similar to HNRNP G)	+	4	1,84	2,08	1,92	4	1,77	1,86	2,14	AV212615	
3110024A21Rik	+	3	1,35	1,88	2,92	5	1,39	2,23	2,34	NM_024215	
6330403K07Rik	+	1	6,23	8,17	10,91	1	3,97	7,32	14,01	AI430680	7
BC031407 (similar to Hs. p66 alpha)	+	3	1,25	2,08	2,92	3	1,12	1,63	3,39	BC038221	
Bhlhb2 (Stra13)	-	10	1,84	2,12	2,27	10	1,31	2,29	2,39	BI686559	5
DAN, Nbl 1	+	4	1,95	2,10	3,03	3	1,62	2,29	3,16	BE956620	
ENSMUSG00000013419	+	2	1,46	1,86	2,53	4	1,43	2,10	1,77	BF456106	
Etv5 (Ets-related transcription factor)	-	6	2,05	2,94	3,90	6	2,77	3,06	2,77	AW825959	
Fosb (FBJ osteosarcoma oncogene B)	-	10	1,72	1,93	2,23	7	1,23	2,14	1,88	AI846927	
H2A histone family member X	-	10	1,86	2,01	1,80	10	1,82	1,65	2,03	AW228881	4, 5, 6
H2A histone family member Z	-	10	1,92	2,16	2,36	10	1,72	2,27	2,29	AI605727	1, 3, 4, 5, 6
H2Bc	+	5	1,55	2,36	2,92	3	1,16	2,39	3,32	AI154951	
HFH-4 (Foxj1)	+	4	1,90	1,86	2,29	4	2,12	2,12	1,95	AW125647	
High mobility group box 2	-	6	1,97	2,29	2,61	10	1,72	2,16	2,27	AI385899	2, 4, 6
Hnrpa1	-	10	1,52	1,72	2,23	10	1,51	1,73	2,05	AA986885	4, 6
Hnrpk	+	4	1,65	2,20	2,20	4	1,75	1,95	2,23	AI528097	6
Inhibitor of DNA binding 4	+	1	2,39	3,86	3,86	1	2,29	3,42	4,06	AI894046	5, 7
Karyopherin (importin) alpha-2 subunit	-	10	2,12	2,08	2,32	6	2,36	2,32	2,51	BG066442	6
Lrrc8	+	5	1,88	3,19	2,53	4	2,34	2,23	2,12	AA620138	
Meiosis expressed gene 1	+	1	3,46	4,31	4,48	1	3,29	4,31	5,00	BF011293	
Oligodendrocyte transcription factor 2	-	10	1,49	1,80	2,29	10	1,57	1,84	2,46	AB038697	2
Paternally expressed gene 3 (Peg3)	-	8	1,54	1,55	2,97	10	1,36	1,84	2,32	W77030	5
Snrp1c	+	4	2,05	2,29	2,72	5	1,95	2,41	2,59	W13021	
Sterol regulatory element binding factor 1	+	4	2,08	2,41	2,64	5	1,80	2,44	2,94	AI386259	
Thymopoietin	-	10	1,54	2,10	2,03	6	1,82	2,64	2,03	AA734516	4, 6
Transcription factor 12	-	10	1,32	1,70	2,03	10	1,34	1,68	2,25	W80054	4, 5, 6
YB-1 (Nsep1)	-	10	1,40	1,75	2,01	10	1,46	1,63	2,25	AI325280	
Zdhhc9	+	5	1,35	2,48	2,05	2	1,42	1,84	2,16	AI317353	
Metabolism											
Brain glycogen phosporylase	+	4	2,36	2,56	2,69	5	2,10	2,77	2,61	BE225750	
Carbonic anhydrase 14	+	2	1,75	1,55	2,36	4	2,14	1,63	1,03	BE954400	
Carbonic anhydrase 2	-	6	1,99	2,23	2,56	6	2,25	2,80	2,86	AK002333	
Cytochrome P450	+	5	2,20	3,03	3,00	5	2,20	3,10	3,29	BE980357	

Dutpase	-	6	2,14	2,16	2,75	6	2,14	2,64	2,75	AI853138	
Glucose-6-phosphate dehydrogenase	+	4	2,14	2,25	2,66	5	2,05	2,89	2,89	AW489197	
Lactate dehydrogenase B chain	+	4	1,60	1,93	2,03	2	1,32	1,84	2,29	AI840631	6
Slc38a3 (NAT-1)	+	1	3,06	3,74	4,48	1	2,14	3,94	4,71	NM_023805	
N-acetylneuraminic acid 9-phosphate synthetase	-	10	1,57	1,80	2,12	10	1,31	1,79	2,14	AI462356	
Oxysterol binding protein-like 5	+	2	1,99	1,72	3,03	3	1,26	2,18	3,29	BE854710	
Phosphoglucose isomerase 1	-	0	1,08	1,77	2,27	0	+ 1,27	1,97	2,83	AW123475	1, 2, 6
Phosphoglycerate dehydrogenase	+	4	1,55	2,12	1,90	4	1,40	2,10	1,65	AK076815	
Prostaglandin D2 synthase	+	5	2,16	3,13	2,08	4	1,82	2,27	2,36	AI323558	5
Thymidine kinase 1	-	6	2,23	2,34	2,56	6	2,10	2,27	2,29	AI325030	6
UDP-glucuronosyltransferase 1	+	4	2,77	2,75	2,01	4	2,64	2,83	1,84	AI043065	
Ion channels / transporters, lipoproteins and steroids											
ATPase, Na+/K+ transporting, alpha 1	+	4	1,88	1,82	2,36	2	1,77	1,99	2,44	W12919	
ATP-binding cassette transporter A3 (Abca3)	+	5	1,68	2,39	2,46	5	1,62	2,44	2,56	AI662539	
Clusterin / apolipoprotein J	+	4	2,12	1,95	2,83	2	1,86	1,80	2,61	W08708	
FXYD1 / PLM	+	1	2,39	4,26	7,85	1	2,34	3,82	7,77	BC024671	
GABA-A receptor, subunit alpha 5	+	4	1,63	1,84	2,10	2	1,26	1,80	2,01	AW046005	
Monocarboxylate transporter 1	+	2	1,17	1,73	2,41	2	1,43	1,93	2,41	AI427128	
Phospholipid transfer protein (Pltp)	+	2	1,36	1,49	2,16	2	1,28	1,54	2,20	AW228730	
Retinal short-chain dehydrogenase/reductase 2	+	5	2,23	2,75	3,10	5	2,01	2,92	3,03	BF020412	
Scavenger receptor class B1	-	10	1,63	2,12	2,77	10	1,34	1,95	3,29	BE996085	5, 6
Sortilin-related receptor	+	2	1,43	1,70	2,20	2	1,23	1,77	2,01	AW261561	
Cell-type specific changes											
Chromogranin B	+	4	1,77	2,34	2,36	5	1,17	2,46	2,20	AA529201	5
Mag (Myelin-associated glycoprotein)	+	3	1,19	1,86	2,89	2	1,23	1,43	2,59	BE992068	
Mbp (Myelin basic protein)	+	3	1,82	2,72	4,85	1	2,08	4,22	4,18	AI844916	
Mobp (Myelin-ass. oligodendrocytic basic prot.)	+	3	1,26	1,90	2,89	2	1,16	1,88	2,14	BE990440	
Neuron specific gene family member 2	+	4	1,99	2,20	2,12	4	1,75	1,97	2,14	U17259	5
Plp (Myelin proteolipid protein)	+	5	1,21	2,36	2,32	2	1,13	1,79	2,61	M15442	
Reticulon 1	+	2	1,60	1,82	2,80	3	1,22	2,23	2,77	BF458469	
RIKEN cDNA 4930565N16 gene (tomosyn)	+	4	2,01	2,44	2,59	5	1,84	2,66	2,80	AK019788	
Syntaphilin	+	2	1,82	1,70	2,64	4	1,57	2,12	1,79	AW045671	
Neural progenitors / differentiation											
Brain fatty acid binding protein (Fabp7)	-	6	2,12	2,56	2,20	10	2,01	1,97	2,66	AI894027	2, 3, 5, 6, 7
Diphtheria toxin receptor (Hb-Egf)	-	6	2,56	3,03	2,20	6	2,14	2,25	1,97	AW491378	5
Neural epidermal growth factor-like-like 2	-	10	1,86	2,12	2,44	10	1,48	2,59	2,83	AI323144	5
Pleiotrophin	-	7	1,09	3,10	4,14	8	1,40	2,61	4,62	CD773543	4, 5, 6, 7
Pacsin3	+	4	1,86	1,99	2,14	2	1,70	1,97	2,46	AI839841	
Syndecan-3	-	7	1,45	2,36	2,59	10	1,60	2,32	2,53	AA050140	5

Table 1. Gene expression changes in neural progenitor cells during differentiation. 161 differentially regulated genes are grouped into ten categories of interest. The trend column indicates an up- (+) or down-regulation (-) during differentiation. Fold changes at 24 h, 48 h, and 96 h compared to the undifferentiated state (0 h) in each experimental series (BDNF and NT4) are given. The cluster numbers are indicated for each gene and experimental series. Accession numbers of representative clones are listed. Numbers in the last column indicate studies that report a comparable expression pattern in neural stem cells. 1-3: genes expressed higher in adult undifferentiated as compared to differentiated neurosphere cells (1: Geschwind et al., 2001; 2: Karsten et al., 2003; 3: Easterday et al., 2003). 4-7: genes enriched in neural progenitor/stem cells in comparison to other stem cell types (4: Ramalho-Santos et al., 2002; 5: Ivanova et al., 2002; 6: Fortunel et al., 2003; 7: D´Amour and Gage, 2003).

Both growth factors, BDNF and NT4, target the TRKB receptor and cause very similar molecular effects in the cells. *In situ*, however, they carry out similar, but not identical functions (Fan et al., 2000). To find gene expression changes that are caused by solely one growth factor, those clones that exceeded a two-fold difference in expression in just one of the two series were inspected. Only those, that presented a two-fold difference in expression at two time points of one series, were examined in more detail. These were only 17 of the 268 clones specific for the BDNF series and only 12 of the 170 clones specific for the NT4 series. They are listed in Table 2. There were no clones exceeding a two-fold difference in expression at all three time points in one and not in the other series. The clones that exceeded a two-fold difference in expression in only one series usually displayed a weaker expression change in the same direction in the other series.

Gene expression changes over two-fold in neural progenitor cells during differentiation in only one series

		Fold Change (BDNF)			Fold Change (NT4)			Accession
Gene	Trend	24 h	48 h	96 h	24 h	48 h	96 h	number
Genes with more than two-fold change in expression in the BDNF series								
Fc receptor, IgG, alpha chain transporter (Fcgrt)	+	1,79	2,44	2,66	1,77	1,68	1,58	W11116
undescribed sequence within clone RP24-243L17 on chr. 4	-	1,99	2,29	2,64	1,63	1,92	1,84	BX004826
Plectin (Plec1)	+	1,60	2,23	2,46	1,57	1,93	1,70	BE648314
Geminin	-	2,61	1,72	2,44	1,51	1,55	1,67	NM_020567
Phosphatidylinositol glycan, class B (Pigb)	+	2,01	1,62	2,32	1,45	1,57	1,68	AI875170
Myelin-associated glycoprotein (Mag)	+	1,12	2,51	2,32	1,21	1,32	1,88	AI893951
Lectin, galactoside-binding, soluble, 3 binding protein (Lgals3bp)	+	1,67	2,56	2,32	1,54	1,95	1,88	AI528677
Breast cancer anti-estrogen resistance 1 (Bcar1)	+	1,58	3,19	2,29	1,39	1,97	1,82	AI848891
U6 snRNA-associated SM-like protein 4 (Lsm4)	-	1,39	2,01	2,27	1,43	1,82	1,77	BF021291
RAB35, member RAS oncogene family (Rab35)	+	1,57	2,39	2,23	1,25	1,88	1,80	AI427439
Deoxyuridine triphosphatase (Dutp)	-	1,79	2,18	2,05	1,79	1,68	1,93	AA537065
Immunoglobulin mu binding protein 2 (Ighmbp2)	+	1,67	2,25	2,03	1,46	1,62	1,63	NM_009212
Microtubule-associated protein 1 A (Mtap1a)	+	1,55	2,59	2,03	1,40	1,77	1,93	AI839626
RIKEN cDNA 2610200G18 gene (2610200G18Rik)	-	2,08	2,39	1,88	1,77	1,72	1,82	AA035912
Thiosulfate sulfurtransferase, mitochondrial (Tst)	+	2,48	2,75	1,65	1,67	1,60	1,75	AW122938
Jun oncogene (Jun)	-	2,25	2,18	1,60	1,90	1,49	1,60	AI528591
Jun oncogene (Jun)	-	2,18	2,03	1,49	1,75	1,39	1,75	AA038998
Genes with more than two-fold change in expression in the NT4 series								
RIKEN cDNA 2410004C24 gene (2410004C24Rik)	-	1,58	1,95	1,63	1,26	2,16	2,80	AA030795
Peroxiredoxin 6 (Prdx6)	+	1,42	1,46	1,40	1,92	2,10	2,46	BE950299
FK506 binding protein 5 (Fkbp5)	-	1,38	1,75	1,99	1,30	2,03	2,39	BC015260
Zinc finger protein 36, C3H type-like 2 (Zfp36l2/Tis11d)	-	1,86	1,63	1,80	1,67	2,20	2,29	AA596105
RIKEN cDNA 0610037L13 gene (0610037L13Rik)	-	1,52	1,84	1,86	1,35	2,05	2,25	AI644347
undescribed sequence within clone RP23-428I5 on chr. 11	+	1,60	1,97	1,80	1,82	2,05	2,18	AL645847
RIKEN cDNA 1700001I21 gene	+	1,99	1,88	1,95	1,82	2,03	2,10	AK005595
Carbonic anhydrase 2 (Car2)	-	1,70	1,90	1,88	1,60	2,03	2,10	AW543133
Histone H3.1-D (H3-D) and Histone H4-D (H4-D) genes	+	1,52	1,19	1,95	2,10	1,70	2,05	MMU62672
Metallothionein 2 (Mt2)	+	1,65	1,30	1,93	2,16	1,92	2,05	AI605645
Hematological and neurological expressed sequence 1 (Hn1)	-	1,49	1,58	1,77	1,73	2,05	2,01	BF467200
Cold inducible RNA binding protein (Cirbp)	+	1,51	1,77	1,73	1,28	2,12	2,01	BF464663

Table 2. Clones showing an at least two-fold difference in transcript abundance at two time points in one experiment (BDNF or NT4), but none in the other series (BDNF or NT4). In the BDNF series, 17 clones met this criterion. In the NT4 series, 12 clones met this criterion. The trend column indicates an up- (+) or down-regulation (-). Fold changes at 24 h, 48 h, and 96 h compared to the undifferentiated state (0 h) in each experimental series (BDNF and NT4) and accession numbers of representative clones are given.

To verify the results of the microarray analysis with an independent method, semi-quantitative RT-PCRs were performed for some of the genes with relevant expression changes. By this means, the differential regulation of gene expression detected by the microarrays was confirmed (Fig. 16).

Figure 16. Semi-quantitative RT-PCR analysis of selected genes. PCR products separated by agarose gel electrophoresis are shown. The respective transcripts were up- or down-regulated as expected from the microarray results. Expression of *Txrnd1* and *Mapk3/Erk-1* was used as a reference.

3.3 *Ptpns1* and *Cd47*

3.3.1 *Ptpns1* and *Cd47* in neurosphere cells

Transcription of *protein tyrosine phosphatase, non-receptor type substrate 1* (*Ptpns1*), also called *Shps-1*, was increased more than four-fold. PTPNS1 is a transmembrane protein and interacts with CD47 (cluster of differentiation 47, also called integrin-associated protein, or IAP) on neighboring cells. This interaction has been demonstrated earlier to be relevant for cellular aggregation and for the migration of immune and melanoma cells (Babic et al., 2000; Liu et al., 2002; Motegi et al., 2003). Interestingly, the expression of *Cd47* also changed during differentiation, as could be demonstrated by semi-quantitative RT-PCR (Fig. 16). Since the differentiation of neural progenitors is linked to their migration *in situ* as well as *in vitro*, the localization of the two proteins in differentiating neurosphere cells was analyzed. Both PTPNS1 and CD47 could be detected in cells within the neurosphere and, to a lesser extent, in migrating cells close to the sphere. Cells that had migrated away from the spheres, however, were negative for both proteins (Fig. 17).

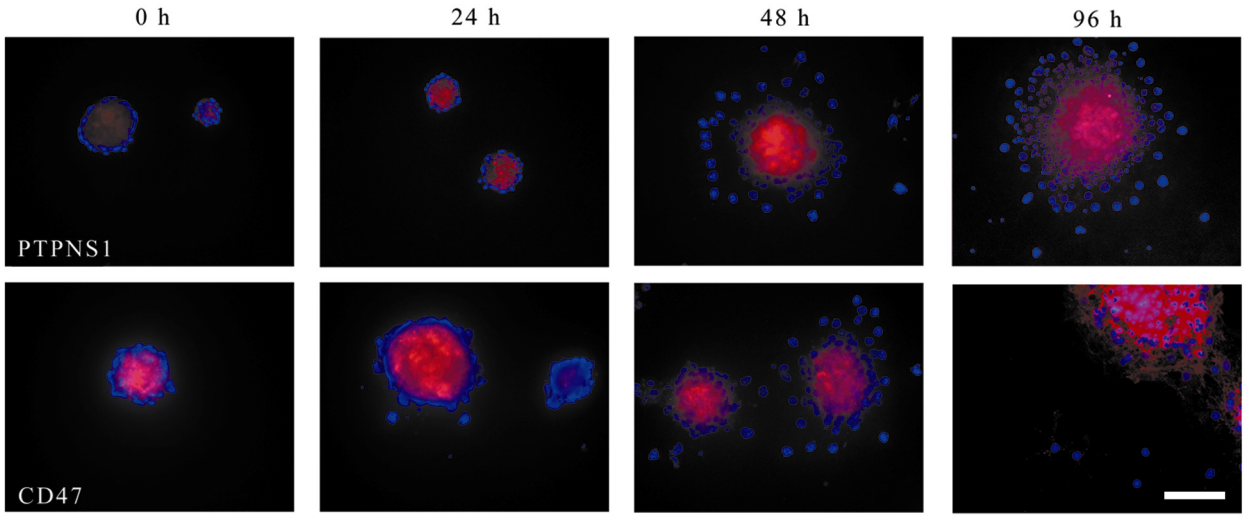


Figure 17. Immunofluorescence microscopy of undifferentiated neurospheres and of cells at 24 h, 48 h, and 96 h after induction of differentiation. Cells were immunolabeled with antibodies against PTPNS1 (upper panel) and CD47 (bottom panel) (both red). Nuclei are stained with DAPI (blue). Scale bar equals 100 µm.

The mode of migration of neurosphere cells *in vitro* differs when the spheres are plated on poly-L-lysine or within an extracellular matrix-like environment, such as Matrigel. Matrigel is a solubilized basement membrane preparation from tumor cells and rich in extracellular matrix proteins such as laminin, collagen IV, and heparan sulfate proteoglycans. In contrast to single migrating cells on poly-L-lysine, in matrigel cells migrated away from the sphere as cellular chains with tight cell-cell contacts (Fig. 18). This type of migration more closely resembles the situation in the rodent rostral migratory stream (RMS), where the neuroblasts also migrate as chains and keep tight cell-cell contacts. These chain migrating cells and the neurosphere cells themselves produced both transmembrane proteins, PTPNS1 and CD47. This observation supports the idea, that these proteins may play a role in neuroblast migration.

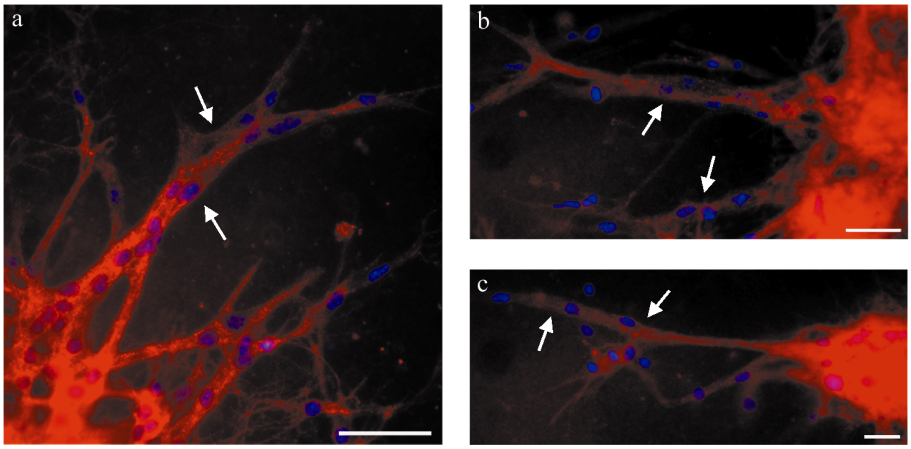


Figure 18. Immunolocalization of CD47 (red in a) and PTPNS1 (red in b, c) in differentiating neurosphere cells that were embedded in Matrigel. DAPI stained the nuclei (blue). Cellular chains are indicated by arrows. Scale bars equal 50 µm.

3.3.2 PTPNS1 and CD47 *in situ*

To study the localization of PTPNS1 and CD47 in the subventricular zone (SVZ) and the RMS, immunohistofluorescence analyses were performed on sagittal brain sections from adult mice (aged three months). PTPNS1 and CD47 were found to be distributed in a similar pattern. In the tissue adjacent to the lateral ventricles, a band of intense staining was detected immediately underlying the less intensely stained SVZ (arrowheads in Fig. 19 a-d). These signals localize to the region that corresponds to the site of contact of the SVZ cells to the extracellular matrix. Weaker signals were detected in what appeared to be the cytoplasm of GFAP-positive astrocytes (arrows in Fig. 19 a, b), demonstrating that some of the progenitor cells produce PTPNS1 and CD47. In addition, some HUC/D-positive cells were also positive for PTPNS1 or CD47 (arrows in Fig. 19 c, d). HUC/D is a nuclear and cytoplasmic protein and is present in early maturing neurons that have left the cell cycle (Marusich et al., 1994; Barami et al., 1995). Thus, both PTPNS1 and CD47 were found to be produced by neuroblasts in the adult subventricular zone.

In the RMS, PSA-NCAM labels the migrating neuroblasts and GFAP labels the surrounding astrocytes (Rousselot et al., 1995; Lois et al., 1996). To analyze the distribution of PTPNS1 and CD47 in the RMS, cells were co-stained for these transmembrane proteins with antibodies against either PSA-NCAM or GFAP. PTPNS1 or CD47 staining partially overlapped with GFAP (arrows in Fig. 19 e, f), but also partially with PSA-NCAM (arrows in Fig. 19 g, h). Thus, both PTPNS1 and CD47 are present in the RMS of adult mice, but it was difficult to attribute protein distribution to specific cell types. To do so, electron microscopy would be required.

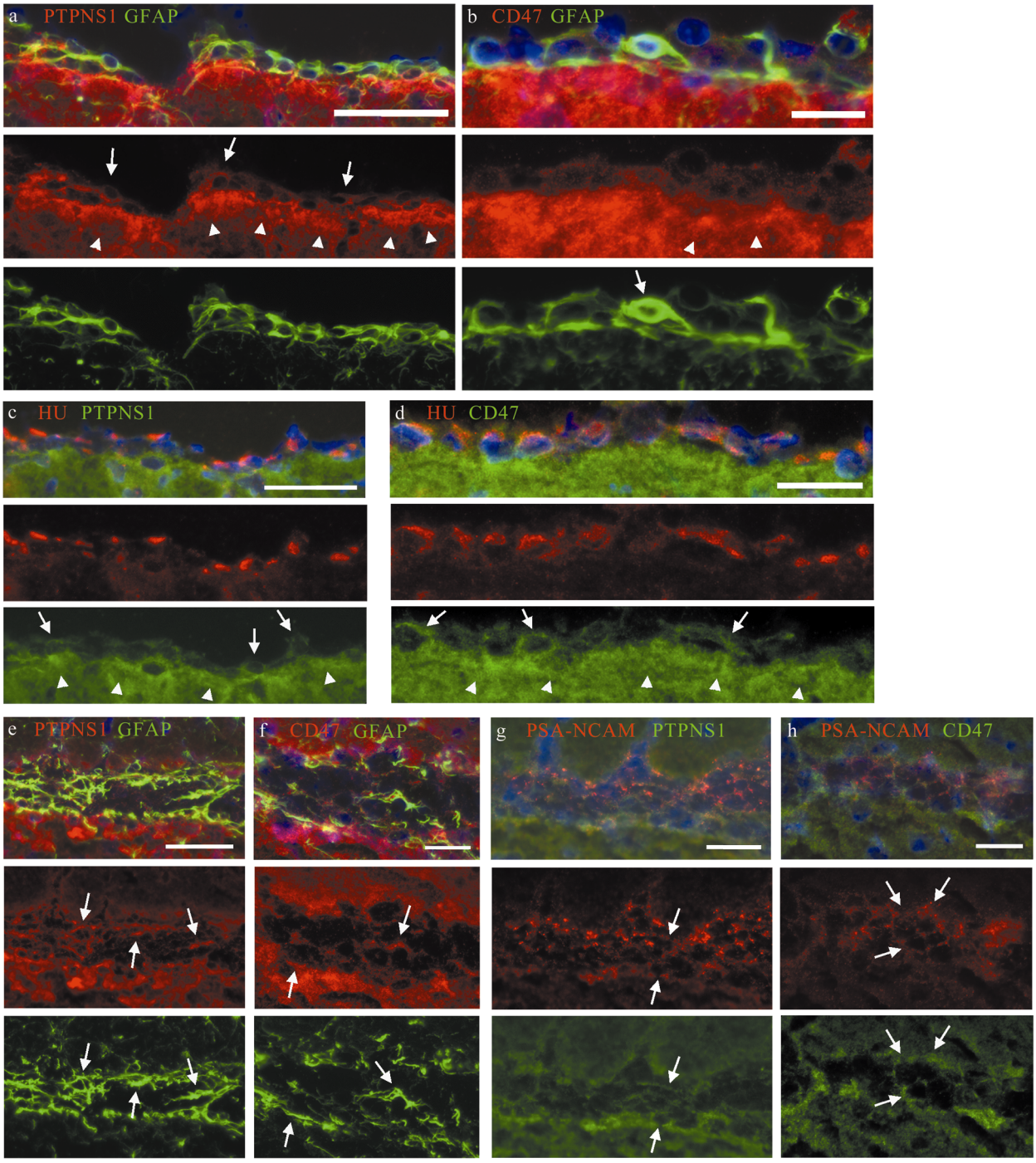


Figure 19. Localization of PTPNS1 (a, c, e, g) and CD47 (b, d, f, h) on sagittal adult brain sections as determined by immunohistofluorescence analysis. Regions of the lateral ventricle wall (a-d) and the RMS (e-h) are shown. Co-immunostainings with GFAP (green in a, b, e, f), HUC/D (red in c,d), and PSA-NCAM (red in g,h) were performed. In c, d, g, h, PTPNS1 and CD47 signals are shown in green. In a, b, e, f, PTPNS1 and CD47 signals are shown in red. Note the band-like PTPNS1 and CD47 staining patterns (arrowheads in a-d). Examples of cells positive for GFAP and PTPNS1 (a) or GFAP and CD47 (b) are marked by arrows. Arrows in c and d depict examples of cells positive for PTPNS1 and HUC/D (c) or CD47 and HUC/D (d). Arrows in e-h indicate an overlapping staining pattern of PTPNS1 and GFAP (e), CD47 and GFAP (f), PTPNS1 and PSA-NCAM (c), as well as CD47 and PSA-NCAM (h). Scale bars equal 50 µm, except for a and e (100 µm).