Therapeutic applicability of

adult brain stem cells

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...all the most acute, most powerful, and most deadly diseases, and those which are most difficult to be understood by the inexperienced, fall upon the brain.

Hippocrates

The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory.

Santiago Ramon y Cajal

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Introduction

Although mitotic figures were described in the wall of the lateral ventricle already in 1912 (1) and the existence of neurogenesis has been suspected as early as the 1960s (2), it was not until 1992 that experimental evidence convincingly proved persisting stem cells in the adult brain (3). This has challenged one of the most intriguing dogmas in neuroscience (4) and was mostly achieved by progress in developmental neuroscience and technical advances in BrdU labeling and cell culturing assays.

The proof of concept for the existence of stem cells residing in the brain and continued neurogenesis into late adulthood has revolutionized the field of neurosciences over the past fifteen years. Adult brain stem cells offer intriguing perspectives for research on brain function and development, CNS disorders and therapy of neurodegenerative diseases. This study will address the potential of adult stem and progenitor cells obtained from rodent and human brain for novel reconstructive therapy strategies.

Terminology

As the term ‘stem cell’ has been used quite promiscuously in many publications, this work will generally follow the terminology used by Gerd Kempermann (5). The most general definition of a stem cell is “a cell capable of both self-renewal and differentiation” (6). An *adult stem cell* is a multipotent cell able to self-renew, i.e. able to generate another stem cell. A *multipotent progenitor* or *transient amplifying cell* is a cell that is derived from a stem cell and is still multipotent, but shows limited self-renewal. Nonetheless, progenitor cells are usually more proliferative than stem cells, while stem cells are often quiescent (5).

In the brain, a progenitor cell is generally more committed than a stem cell, e.g. being able to produce astrocytes and oligodendrocytes, but not neurons. A precursor is any cell not fully differentiated, thus the term “precursor cell” includes both stem and progenitor cells.

Neurogenesis refers only to the *in vivo* proliferation of precursor cells that differentiate into new neurons and this term should not be used for *in vitro* observations.

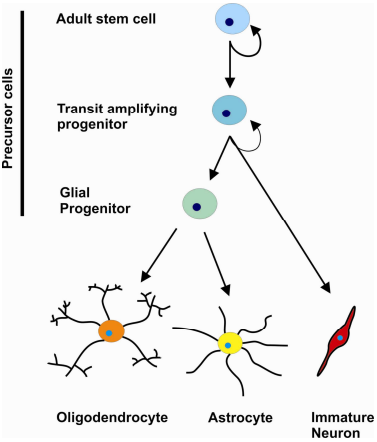


Figure 1: Hierarchy of adult CNS stem cells. Adult stem cells are self-renewing multipotent cells that give rise to transit amplifying progenitors (whose minor self-renewal capabilities are reflected by a thinner arrow). Precursors encompass stem and progenitor cells. Multipotency in the CNS reflects the ability to generate oligodendrocytes, astrocytes and neurons. Modified from (7).

Stem cells and 'stemness'

The two most noteworthy characteristics of adult stem cells are unlimited self-renewal and multipotency (8). Multipotency is the ability to give rise to at least two different cell types (5). However, the term may also refer to the capability of producing all cell types of a certain organ. The latter is the context in which multipotency is used in this study. While adult stem cells of the brain are truly multipotent *in vitro*, it has to be noted that the *in vivo* situation is quite different. *In vivo*, most adult stem cells differentiate into neurons, while a very small part may differentiate into astrocytes and oligodendrocytes (9).

One of the major hallmarks of stemness, self-renewal is the ability of a cell to produce at least one daughter cell with the same features as the mother cell upon division. This can be achieved either by symmetric or asymmetric division. Symmetric division of a stem cell yields two stem cells and mostly occurs in the stem cell expansion phase during embryogenesis. Asymmetric division results in one stem cell and one more committed progenitor cell. This progenitor cell can also be multipotent and have the ability for self-renewal. In contrast to the originating stem cell, multipotent progenitor cells have restricted self-renewal capabilities. Asymmetric division has been identified as mechanism for SVZ neurogenesis (10): inclination of the cell division axis results in unequal distribution of the apical membrane, which defines stem cell and committed progenitor cell. Conceivably, symmetric division can also result in terminal differentiation, yielding two committed progenitors. However, this would result in depletion of the stem cell pool. Notably, the total number of adult stem cells within the brain declines with age, while the mechanism of this decline remains unclear (11). Another study, however, did not observe reduced numbers of stem cells in aged rodents (12).

Neuronal development during embryogenesis

The process of adult neurogenesis is closely related to the development of neurons during embryogenesis, as several signaling pathways responsible for the generation of neurons during embryogenesis are also relevant for adult neurogenesis (13). Adult stem cells of the subventricular zone are thought to be derived from radial glia cells, which are the source of neurons in embryonal development (14). During the development of the central nervous system, the earliest neural stem cells form at the walls of the primordial brain ventricle. The ventricular walls are germinative regions throughout life (5). Radial glia are located at the ventricular zone and are thought to be the stem cells of the developing nervous system (15-17). However, the existence of an independent stem cell pool cannot be excluded at the moment. By asymmetric division, radial glia give rise to neuroblasts, which use the processes of radial glia to migrate longitudinally towards the surface of the cortex (18). The cortex is layered in an inside-out fashion: the first cells produced forming the innermost layers of the cortex (18). Reelin, an extracellular matrix protein produced by Cajal-Retzius neurons of the cortex, stops the migration of neuroblasts (5). Later during development, radial glia produce astrocytes. Adult stem cells of the SVZ are most likely remnants of radial glia cells (14).

Adult neurogenesis

Multipotent adult precursor cells can be isolated from a great number of brain areas (19-22). However, neurogenesis occurs only in two different areas of the brain, the subventricular zone (which is a derivative of the embryonic ventricular zone) and the dentate gyrus of the hippocampus (23). There have been reports claiming neurogenesis in other brain areas, e.g. cortex (24), substantia nigra (25) and the striatum (26), but these reports remain controversial and neurogenesis is in these regions most likely a reaction to tissue damage.

Neurogenesis is the process by which stem cells divide (proliferation) and give rise to neuronal precursor cells (neuroblasts) that migrate to a specified destination (migration and differentiation), where they mature into neurons and integrate into brain circuitries (synaptic integration). Whether precursor cells isolated from other brain regions are true stem cells may be debated, as these cells react like stem cells *in vitro*. Neurogenesis has been proven only in the two regions stated above. The time-course for the production of a fully integrated neuron from a stem cell is approx. 4 weeks in adult rodents (23). It has been estimated that in the rat hippocampus 9,000 new neurons are born *per die* (27). Of course, a large part of the newborn cells are eliminated by apoptosis and only some cells will become integrated neurons (28). Yet, newborn neurons do not contribute to neuronal turnover and the rodent hippocampus grows throughout life, even if postnatal growth is too minute to measure (5).

The subventricular zone

In the SVZ, stem cells reside directly behind the ependymal cells and some of these stem cells have contact to the ventricular lumen via

small processes (29). The cell types of the SVZ have been characterized by electron microscopy (29) and stem cells have been identified as the 'type B' cells that express GFAP (30). SVZ stem cells give rise to rapidly dividing, transit-amplifying precursor cells ('type C') that are still multipotent, but lack the ability to self-renew (30). Type C cells will differentiate into neuroblasts ('type A'), which migrate along the so-called rostral migratory stream (RMS) and integrate into the olfactory bulb (31). Neuroblast migration has been described as 'chain-migration' (32), as neuroblasts are migrating in single file and are ensheathed by processes of GFAP-positive astrocytes (31). Having reached the olfactory bulb, neuroblasts will terminally differentiate and integrate into the olfactory circuitry either as periglomerular neurons or as granule cells (23). Type B cells also produce oligodendrocytes *in vivo*, although to very small percentages (9).

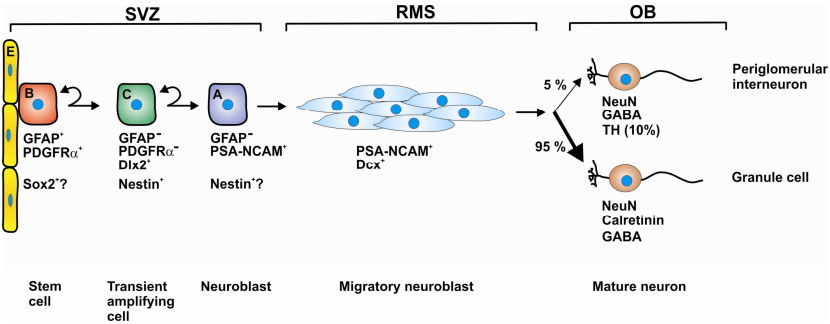


Figure 2: Subventricular zone neurogenesis. SVZ stem cells (type B) reside directly behind the ependymal layer (E) and are characterized by GFAP and PDGFRα expression. Some also express Sox2. Stem cells produce transient amplifying progenitor cells (type C) that are GFAP and PDGFRα negative, but express Dlx2 and Nestin. Type C cells differentiate into neuroblasts (type A) that are defined by PSA-NCAM expression, while Nestin levels diminish. Type A cells begin to migrate along the RMS, where they are called migratory neuroblasts. Migrating cells can be identified using PSA-NCAM and Dcx. Neuroblasts reaching the olfactory bulb mature and integrate into neuronal circuitry. The largest part will become GABAergic granule cells (expressing NeuN, and calretinin), while some cells differentiate into periglomerular neurons (defined by expression of NeuN and either GABA or – to a small part – TH).

In the human SVZ, stem cells seem to reside not directly behind the ependymal layer, but approximately one cell layer beneath (33). The human equivalent of the rostral migratory stream has been recently identified (34).

The hippocampus

The second region of adult neurogenesis is the hippocampus. As the true nature of multipotent hippocampal progenitor cells remains controversial (35-37), they will be referred to as hippocampal precursor cells throughout the text. Multipotent precursor cells have been identified in the cell layer directly beneath the granule cells in the dentate gyrus, which has been called subgranular zone in analogy to the SVZ (8). These cells also have a radial glia-like morphology and are thus referred to as type B cells (38). The progeny of type B cells are rapidly dividing progenitor cells (transit-amplifying cells) that in the hippocampus are called 'type D' cells (39). These cells will differentiate into neuroblasts that migrate a short distance into the granule cell layer where they express calretinin and integrate as granule cells into the dentate gyrus. The new-born granule cells will send axons into the CA3 region of the hippocampus and receive synaptic input from the molecular layer of the dentate gyrus. Mature granule cells are characterized by calbindin expression.

In the hippocampus, stem cells reside in close proximity to microvascular vessels (40) that provide the stem cells with growth factors such as VEGF (41, 42). This microenvironment has been termed the stem cell 'niche' (40).

The groundbreaking work of Eriksson et al. described the process of adult neurogenesis in the human hippocampus and was indeed the first publication to prove the existence of human neurogenesis (43).

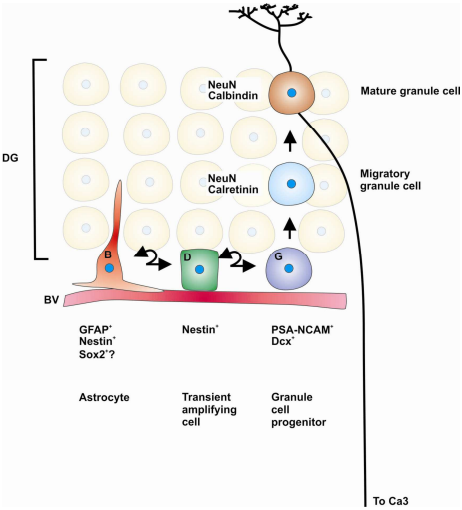


Figure 3: Hippocampal neurogenesis. Multipotent astrocytes (A; GFAP and Nestin positive, some expressing Sox2) reside below the granule cell layer in close proximity to blood vessels (BV). These astrocytes produce transient amplifying progenitor cells (type D) that are expressing Nestin but not GFAP. These differentiate into granule cell progenitors (G) that express PSA-NCAM and Dcx. The granule cell progenitors that migrate into the dentate gyrus can be identified by expression of NeuN and calretinin, while mature granule cells that extend their axons to CA3 express NeuN and calbindin.

Physiological impact of neurogenesis

Neurogenesis is a widespread, evolutionally conserved phenomenon in the animal kingdom that has been described in several vertebrate species, including songbirds, rodents, zebrafish and humans (2, 43-45). Interestingly, bats are the first mammalian species in which no neurogenesis within the hippocampus could be identified (46). On the other hand, this study relied only on doublecortin staining and did not include BrdU labeling for the identification of proliferating precursor cells differentiating into neurons. Hence, the existence of neurogenesis cannot be completely excluded.

The most important indicators of the possible physiological impact of neurogenesis in adult mammals are the parameters modulating neurogenesis. In the hippocampus, neurogenesis is enhanced by 'environmental enrichment', an experimental paradigm in which animals are housed in small colonies and in enlarged cages, while having access to toys, running wheels and tunnels. The context of the environment is changed frequently. Under these conditions, hippocampal neurogenesis increases measurably and animals housed in enriched environments perform significantly better in hippocampal learning and memory tests, such as the Morris water maze (47). This points to a function in memory and spatial orientation for hippocampal neurogenesis (48). On the other hand, reports that hippocampal neurogenesis is not necessary for the behavioral effects of environmental enrichment (49, 50) bring any prominent role of neurogenesis for spatial learning and orientation into question. Thus, enhanced learning capabilities might increase hippocampal neurogenesis, but not vice versa. Alternatively, neurogenesis might be necessary for some experimental settings, but not for others (51). A third possibility is that the different markers used in the above-mentioned studies may have influenced the reported findings.

While enriched environment has no effects on SVZ neurogenesis (52), an experimental paradigm that increases subventricular zone neurogenesis is ‘odor enrichment’ (53). Animals that are exposed to frequently changing and different odors have an increased number of neurons integrating into the olfactory bulb and show an improved olfactory memory (53). Thus, it seems that continued production of neurons is important for continuing brain plasticity during changing environment (54). In humans, only circumstantial evidence exists that may point at physiological functions of neurogenesis. Increased hippocampal volume as measured by MRT correlates with working experience in London’s taxi drivers, who need to have good spatial orientation (55).

In Parkinson’s patients, SVZ neurogenesis is decreased (56) and the olfactory bulb is one of the brain regions in which neuropathological correlates of the disease can be first identified (57). Moreover, a decreased sense of smell has been found to be an early symptom during the prodromal stage of Parkinson’s – long before any motor symptoms appear (58).

Differentiation of adult stem cells

Cell lineages

Adult neural stem cells are able to differentiate into the three major CNS cell populations, i.e. astrocytes, neurons and oligodendrocytes. Astrocytes and oligodendrocytes are referred together as 'glial lineage' in contrast to the 'neuronal lineage'. Multipotent precursor cells eventually differentiate into lineage committed progenitor cells that are still able to differentiate into several cell types of one lineage (e.g. glial-restricted progenitors that can differentiate either into astrocytes or oligodendrocytes). These differentiate into even more committed progenitor cell stages that finally terminally differentiate (e.g. oligodendrocyte precursor cells that become myelinating oligodendroglia).

Macroglia

The glial lineage consists of astrocytes (both type I and type II), oligodendrocytes and ependymal cells. Further classification of oligodendrocytes into light, medium-shade or dark cells may rather reflect different stages of maturity (59). Oligodendrocytes are immunocytochemically identified by expression of CNPase, GalC, Rip, O4, MBP or BLBP (59). Type I astrocytes appear to arise directly from multipotent progenitors, while Type II cells derive from O2A progenitors, which are also oligodendroglial precursors (hence the name "oligodendrocyte-type 2 astrocyte-progenitor"). Astrocyte markers include GFAP, S100β, GLT-1 and glutamine synthetase (59). It is unclear whether adult stem cells can differentiate into ependymal cells *in vitro* or *in vivo*. While adult ependymal cells were identified as postmitotic (60), their expression of the stem cell marker prominin-1 (CD133) (61) may indicate that ependymal cells are rather quiescent

than terminally differentiated. However, this awaits further analysis. Ependymal cells can be identified by their multiple apical cilia and by expression of tubulin beta IV or S100 (61).

To date, few common ancestral cells are identified (both for the neuronal and the glial lineage) whereas most cell types between the adult stem cell and the latest progenitor cell stages remain poorly characterized.

Neurons

Neurons are classified according to their morphology (e.g. pyramidal cells, granule cells, Purkinje cells), anatomical location (e.g. cerebellar neurons, cortical neurons), transmitter phenotypes (e.g. GABAergic, serotonergic, dopaminergic), electrophysiological properties (e.g. spiking or bursting neurons) or the functional circuitry to which they belong (e.g. interneurons, motor neurons, sensory neurons, association neurons). Given the abundant variety of neuronal cell types, it is striking that only a handful of neurons is produced *in vivo*. These are periglomerular interneurons and granule cells in the olfactory bulb and hippocampal granule cells, respectively (23). SVZ stem cells differentiate into GABAergic and dopaminergic neurons while stem cells from the hippocampus produce GABAergic and glutamatergic neurons (23, 62). Isolated stem cells do not retain their regional profile *in vitro* (63) and heterotypic grafting of hippocampal stem cells into the RMS resulted in olfactory neuron differentiation (64). Thus, stem cell differentiation largely depends on the microenvironment.

Tubulin beta III, PSA-NCAM, Dcx and Map2 identify immature neurons, while mature neurons express neurofilament, NeuN and metabolic enzymes for neurotransmitter synthesis (such as tyrosine hydroxylase or glutamic acid decarboxylase) (5, 23, 65-67).

Epigenetic contributions to stem cell fate decision

Stem cells produce committed progenitor cells, which become lineage-restricted during their maturation. Some molecular pathways of this lineage-restriction have been identified. Precursor cells are pushed into a certain lineage by soluble factors, cell-cell or cell-matrix interactions. A growing number of factors have been identified capable to drive the differentiation of stem cell progeny towards specific fate determination (68-76). Also, environmental cues can trigger cell-intrinsic pathways of chromatin remodeling that will result in large changes of gene expression needed for differentiation along different lineages (77). Recent studies have shown, however, that intrinsic mechanisms of epigenetic chromatin remodeling significantly induce cell fate determination in the CNS (78-82).

Mechanisms of epigenetic regulation

Expression of genes and translation into proteins is regulated by multiple mechanisms. Transcription factors acting in *trans* can bind to certain sequences of the DNA and either activate or repress transcription. Gene transcription can also be indirectly regulated by conformational changes of the DNA. The combination of DNA and the histone proteins facilitating nucleosomal packing is called chromatin. Chromatin can either have a relaxed (loose packaging of DNA with light binding of histones to DNA) or condensed (tight packaging of DNA with intense binding of histones to DNA) conformation. Condensed chromatin (heterochromatin) is in a “closed” state, thus restricting transcription factor binding sites on the DNA. In contrast, relaxed chromatin (euchromatin) facilitates the binding of transcription factors and thus, gene expression. The configuration of chromatin can be influenced in two ways: methylation of cytosine residues on the DNA or post-translational modification of histones, which influence each other (83).

Methylation of cytosine residues in DNA occurs predominantly at repetitive CG elements commonly called “CpG-Islands”, but is not restricted to these sites. These islands are 5´-CG-3´ dinucleotide repeat sequences, often located at the 5´ regions of genes (84). In mammalian DNA, approximately 2% to 5% of all cytosine residues are methylated (85). In CpG–Islands, often multiple consecutive cytosines are methylated.

Post-translational histone modifications include the histone H2A, H2B, H3 and H4 proteins of the histone octamer and may consist of serine/threonine phosphorylation and lysine acetylation or methylation. In addition to the type of modification, also the position of the modified residue is important – this has been called the “histone code” (86, 87). Many histone-modifying enzymes interact with either trans-activating or trans-repressing protein complexes or with the DNA replication machinery, depending on the function of the histone-modifying enzyme (88).

As DNA-methylation is a rather slow process (and seems almost irreversible in many cells) (89), it is prone to regulate long-term changes in chromatin structure, such as the shutdown of gene clusters that are not required for cellular function. Histone modifications underlie a much faster turnover, and are relevant for regulation of genes that need to be expressed context-dependently. For example, organ-specific genes that have no relevance in the CNS will be turned off by DNA methylation in all CNS cells. On the other hand, CNS-specific genes may be silenced by histone modifications in adult CNS stem cells and either be further shut down by methylation (if un-needed) or activated by changes in histone modifications if their expression is required (Figure 4).

Acetylation is facilitated by histone acetyltransferase (HAT, E.C. 2.3.1.48) and can be reversed by histone deacetylase (HDAC, E.C. 3.5.1.-). In addition to histone acetyltransferases, there are some transcription factors that also have HAT activity, such as p300/CBP and

P/CAF. These transcription factors provide a link between established signal transduction pathways (e.g. BMP/TGFβ, Notch, Jak/STAT) and chromatin remodeling.

Histone deacetylases are divided in 3 categories (90). Class I HDACs (HDAC 1, 2, 3 and 8) are homologous to yeast Rpd3, with the catalytic domain in the central region of the protein and a molecular weight of roughly 50 kDa. The class II enzymes (HDAC 4, 5, 6, 7, 9 and 10) have a high homology to yeast Hda1, a size of about 120 kDa and a catalytic domain near the C-terminus. Unlike class I enzymes, class II HDACs are able to shuttle between the nucleus and the cytoplasm, which seems to be facilitated by their binding to certain proteins (91). Class III HDACs (SIRT 1 – 7) share homology to Sir2 proteins in yeast and are NAD^+-dependent. All HDACs share a common region of 390 amino acids, which is called the deacetylase core (92). Class I HDACs are expressed ubiquitously, while the class II enzymes are confined to heart, skeletal muscle and brain (93).

The involvement of histone acetylation has been shown in the differentiation of myocytes (94) and is most likely also true for the development of the CNS (78, 80). One key mechanism of HDAC inhibitors is the induction of cell cycle inhibitors, e.g. $p21^{WAF}$, thus initiating differentiation (95).

One common gene expression regulatory element is the neuron-restrictive silencer factor (NRSF, also known as RE1 silencing transcription factor, or REST), which interacts with HDACs and recruits these enzymes to the response element 1 (RE-1 or neuron-restrictive silencer element, NRSE) in non-neuronal cells (96, 97). Thus, neuronal gene expression is shut down in CNS cells other than neurons.

In neuronal committed progenitors, a small, non-coding dsRNA containing the NRSE sequence is expressed. This dsRNA interacts with

NRSF, removing the silencer from neuronal gene promoters (98). As HDACs are a requisite component of these silencing clusters, inhibition of HDAC enzymes may also result in neuronal gene expression.

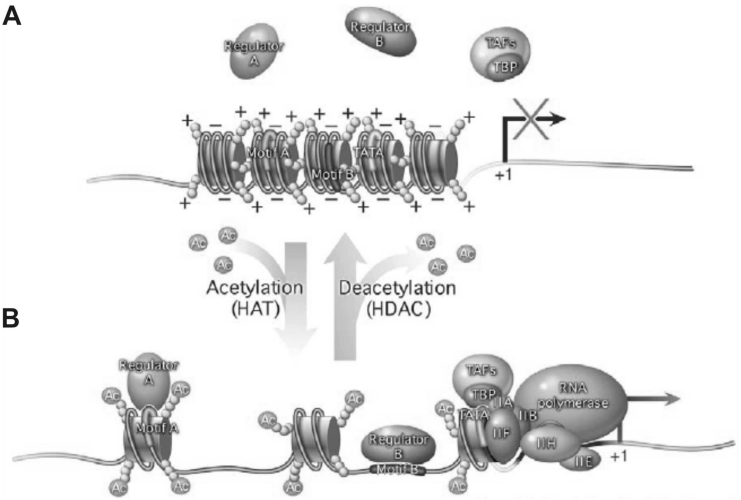


Figure 4: Epigenetic regulation of gene expression. A, heterochromatin is transcriptionally silent. Histone molecules are deacetylated at certain lysine residues. Acetylation of these residues promotes formation of euchromatin. **B**, Euchromatin is transcriptionally active. Histone lysine residues are acetylated. *Trans*-regulatory elements can bind to the DNA and genes are expressed. Adapted from (77).

Histone deacetylase inhibitors: Tools for influencing epigenetic regulation

HDAC inhibitors are classified in five subgroups according to their chemical properties: aliphatic acids (e.g. phenylbutyrate, VPA), hydroxamic acids (TSA, SAHA, M344, CBHA, SBHA and others), cyclic tetrapeptides (e.g. apicidin, depsipeptide), electrophilic ketones and benzamides (e.g. MS-275). It has been shown that HDAC inhibitors induce growth arrest, differentiation, inhibition of proliferation, and apoptosis in various tumor cell lines. These properties suggest also anti-tumor efficacy of HDAC inhibitors (92, 99) and some drugs are already

under investigation in clinical phase II/III trials (e.g. depsipeptide) (91). Suberoylanilide hydroxamic acid has been FDA approved in late 2006 for treatment of cutaneous T-cell lymphoma under the generic name Vorinostat (Merck & Co). Hydroxamic acid compounds appear more selective for HDAC 1 and HDAC 3, which they inhibit in a nano- to micromolar range (92). Hydroxamic acids are also the most potent HDAC inhibitors known to date, showing activity at nanomolar levels. The least potent inhibitors are aliphatic acids with concentrations at low millimolar levels (91).

HDAC inhibitors appear to increase the number of acetylated proteins and promote transcriptional activation (91). Transcriptional activation following HDAC inhibitor treatment is pleiotropic, but affects only a moderate number of genes (Dr. E. Hahnen, ZMMK Cologne, personal communication). Recent estimates indicate that 2 % to 5 % of the genome is either activated or repressed (91).

Histone acetylation plays a key role in cellular differentiation. While HATs activate expression of differentiation-specific genes, HDACs repress the cell cycle and proliferation-specific genes. With the inhibition of HDACs, but continuing HAT activity, there is an overhang of histone acetylation leading to an increased expression of genes for differentiation (91).

Stem cells *in vitro*

Besides being identified *in vivo*, stem cells of the adult brain can also be isolated and cultured *in vitro*. Unlike in the hematopoiesis field only very few surface antigens have been identified in neural stem cell research. Thus, proof of stemness relies mainly on cell culture assays. First described by Reynolds and Weiss (3), the Neurosphere Assay remains one of the most rigorous tests for neural stem cells *in vitro* to

date (Figure 5). In the Neurosphere Assay, stem cells are placed into viscous medium (consisting of 1.2 % methylcellulose) in the presence of growth factors (usually EGF and FGF-2) at a density less than 10,000 cells/cm^2. These culture conditions are regarded as clonal (meaning that individual cells cannot interact with each other) and inhibit the growth of most cells. Only stem cells and some multipotent progenitor cells will proliferate in these conditions, forming globular clusters of hundreds to thousands of cells, which are called neurospheres. These neurospheres can be dissociated and replaced into the same culture conditions, where they will form spheres again, which are called secondary spheres. Only cells that can give rise to primary and secondary spheres under these rigorous conditions and that are truly multipotent are *bona fide* stem cells. To test for multipotency, individual spheres (that arose from a single cells) are picked and placed into permissive conditions where cells can differentiate – normally consisting of adhesive surfaces, withdrawal of growth factors and addition of serum. The progeny of a neurosphere can then be identified as the cells surrounding the original sphere and immunostained for markers of neuronal and glial lineage. Stem cells form multipotent neurospheres, differentiating into astrocytes, oligodendrocytes and neurons. However, proof of multipotency is generally accepted when neurosphere-derived cells produce cells from both the neuronal and glial lineage.

Contrary to the situation *in vivo*, great differences exist when stem cells are differentiated *in vitro*. While the main purpose of stem cells *in vivo* seems the production of new neurons (100), *in vitro* these cells also differentiate into astrocytes and oligodendrocytes in large quantities. Furthermore, *in vitro* multipotent progenitor cells can be isolated from almost all brain regions, including the striatum, spinal cord, substantia nigra, cortex and the cerebellum (19-22).

It has been claimed that this finding reflects the ability of some astrocytes *in vitro* to differentiate into neurons and that some astrocytes are multipotent (101). However, the physiological role of such cells is

controversial, as well as the question whether some or all astrocytes are multipotent *in vitro* (102-104).

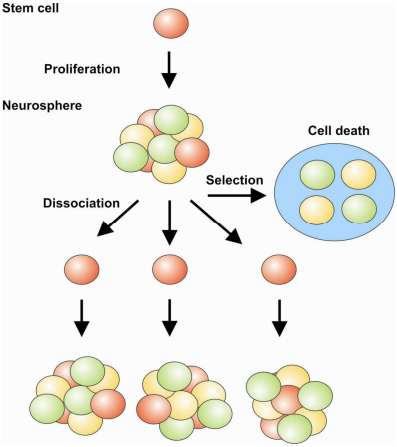


Figure 5: The Neurosphere Assay. Stem cells proliferate *in vitro* when placed into suspension culture conditions and give rise to neurospheres. These neurospheres are comprised of a mixture of stem and progenitor cells. Upon dissociation and re-plating ('passaging'), the more committed progenitor cells die, while the stem cells give rise to new ('secondary') neurospheres. Modified from (105).

Stem cells and diseases of the CNS

Neurogenesis is a physiological process, which may be disturbed in some disorders. On the other hand, the dysfunction of neurogenesis may by itself cause disease. The number of diseases in which a change in neurogenesis occurs is increasingly becoming recognized (106-110). Moreover, an increase in stem cell proliferation and migration towards damaged tissue has been observed as reaction to several CNS injuries, e.g. stroke and tumors (111, 112).

Among the disorders in which some alteration of neurogenesis has been found are Alzheimer's disease (107, 113), mesial temporal lobe epilepsy (108, 114), depression (106, 115), Huntington's disease (Dr. S. von Hörsten, Experimental Therapy, University Erlangen; Dr. L. Aigner, Dept Neurology, University Regensburg; and F. Siebzehnrübl, unpublished observation)(116) and Parkinson's disease (109, 110). While the correlation between the pathophysiology of these disorders and the changes in neurogenesis is largely obscured, it is clear that neurogenesis is a complex process that is regulated by a diverse spectrum of factors.

In Parkinson's disease, dopaminergic neurons within the substantia nigra degenerate, thus leading to the classic motor symptoms of the disease. These neurons innervate large areas of the brain, including the subventricular zone. The observation that SVZ neurogenesis is decreased in Parkinson's disease has led to the identification of dopamine as a regulator of stem cell proliferation (56).

Depression is caused by a deregulation of the serotonergic system, serotonin being another neurotransmitter regulating neurogenesis (117), whereas antidepressant treatment takes several weeks before effects can be seen – a time span which may be explained by the need for new cells

to grow and integrate into neuronal networks. Furthermore, the fact that most antidepressants tested influence stem cell proliferation and that these substances have no effect *in vivo* when neurogenesis is ablated has led to the hypothesis that depression may be a "neurogenesis disorder" (106, 117-119).

In mesial temporal lobe epilepsy (MTLE) an increase of proliferating neural precursors within the hippocampus has been observed (108, 114, 120). The hippocampus is the structure where the focus of epileptic seizures lies in MTLE and also where most of the damage occurs. Whether the increase of hippocampal neurogenesis is a cause of the disease or a reaction to it remains to be shown (121).

Finally, in Alzheimer's disease, an increased hippocampal precursor proliferation also has been observed (113). Notably, the hippocampus is one of the structures affected first in the earliest stages of AD (122) and the amyloid precursor protein (APP; the precursor to the protein that is accumulated in amyloid deposits in the brain in AD) is physiologically expressed in neurogenic regions of the brain, i.e. the SVZ and the hippocampus, and impacts stem cell proliferation and differentiation (123-125). However, a physiologic function of APP has not been identified to date.

Regenerative stem cell medicine

The possibility of isolating expandable cells able to differentiate into cell types ablated in neurological disorders has sparked hopes for therapy of these hitherto incurable diseases. Potential therapeutic applications of stem cells include transplantation of stem cells or stem cell-derived progenitor cells, grafting genetically modified cells replacing required transmitters or growth factors and changing brain microenvironment to become permissive for endogenous neurogenesis (126). Depending on the strategy pursued, different kinds of disorders are candidates for stem cell-based therapies. When grafting stem cells that will differentiate and replace lost cells, disorders with focal cell loss (e.g. Parkinson's, Multiple Sclerosis, but also stroke or spinal cord trauma) are likely to benefit from such therapy strategies. However, if grafted cells are modified to produce certain growth factors, other diseases may be treated as well. These include Alzheimer's disease, amyotrophic lateral sclerosis and Huntington's disease (127, 128). Interestingly, enhanced proliferation and differentiation of endogenous stem cells has been observed in rodent brains following ischemic lesions (129). Whether human stem cells also proliferate following stroke and whether these endogenous stem cells have the capacity to support recovery after stroke remains to be shown. However, the time-interval for recovery after stroke is in favor for a cellular response.

Several experimental therapeutic attempts in disease models have already been undertaken, however, no (pre-) clinical studies using adult brain stem cells have yet been initiated. In one of the first approaches, neurospheres were administered intravenously in a mouse model of Multiple Sclerosis (130). Surprisingly, the neurospheres homed to the CNS, integrated into the host brain and led to a relief of symptoms.

Several possibilities for stem cell based therapies are conceivable: A) Transplantation of embryonic stem cells. As ES-cell derived grafts have been shown to induce tumors upon transplantation (131) and are ethically precarious, this option will not be further discussed. B) Transplantation of transdifferentiated neuronal precursor cells derived from bone marrow or umbilical cord stem cells. The ability of stem cells to transdifferentiate is still controversely discussed (132, 133), so this option is questionable at the moment. C) Autologous transplantation of patient-derived SVZ stem cells. Autologue grafts have the advantage of being immunocompatible. However, this option depends on the pre-existing condition of the patient. D) Allografting an adult neural stem cell line. The stem cell line in question would not necessarily have to be immunocompatible, as the brain is an immunoprivileged organ. However, microglial cells can produce some immunoreactions and there are very few studies on functional long-term grafts available. E) Grafting an adult stem cell line that will produce growth factors. Cell grafts actively producing trophic factors have the advantage of continuous release at the site of demand. F) Activation of endogenous brain stem cells to proliferate and differentiate. This alternative may either replace or support the above-mentioned options.

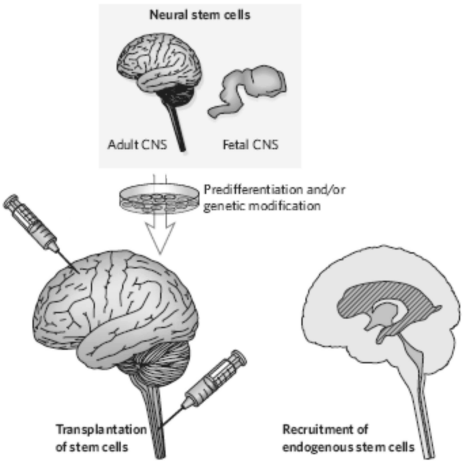


Figure 6: Therapeutic transplantation of stem cells for neurological diseases. Transplantation of stem cells derived from the fetal CNS has been performed. Adult hippocampal precursor cells may be transplanted (with or without preceding genetic modification) and replace lost CNS cell types. Alternatively, endogenous stem cells may be activated to proliferate and differentiate into desired cell types. Modified from (128).

Cancer stem cells

The cancer stem cell hypothesis is increasingly recognized as current model to understand tumorigenesis (134, 135). In contrast to the stochastic model of tumor growth and metastasis, where basically each tumor cell is able to drive tumor growth and the likelihood of metastasis is governed by chance and probability, the hierarchical model proposes cancer stem cells as sole culprits for continued growth of tumors and the formation of remote tumor clones (136). Cancer stem cells have self-renewal capabilities and produce more differentiated tumor cells, which continue to proliferate for a limited time period (though not indefinite) but cannot produce metastases upon spreading (137).

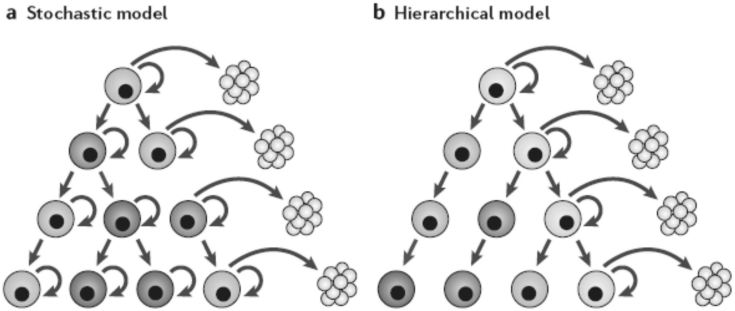


Figure 7: The cancer stem cell hypothesis. A, in the stochastic model, all cells may produce tumor clones, depending on stochastic probability. **B**, in the hierarchical model, only cancer stem cells are able to produce tumor clones, while all other tumor cells are more differentiated. Adapted from (136).

The cancer stem cell hypothesis explains some of the typical properties of tumor growth. For example, metastasis is an exceedingly rare process when compared to the number of tumor cells abound. This may be explained when only cancer stem cells are able to form new tumor clones and these stem cells are a small subpopulation of the whole tumor.

Cancer stem cells have been identified in a variety of malignancies, e.g. leukemia (138), CNS tumors (139-141) as well as colorectal and prostate carcinomas (142, 143). Although the emergence of malignant brain tumors from persisting stem cells has been suspected, no direct evidence has been found identifying a founder (stem or progenitor) cell in these tumors.

Repetitive isolation of stem-like cells from brain tumors (139, 140) has re-entered the intriguing discussion from which cell population these tumors derive. Stem and progenitor cells from the subventricular zone have long been suspected as potential source of malignant gliomas (144). Current hypotheses favor, therefore, that stem cells acquire mutations leading to cell cycle deregulation or dedifferentiation of more committed progenitor cells, which result in cancer founding stem cells. Pinpointing which exact cell type (i.e. stem cell or progenitor) is the origin of a tumor remains, however, one of the key issues in cancer stem cell biology.

Recent discussions addressed a transforming potential of adult neural stem cells (144), a property that has already been shown for adult mesenchymal stem cells (145, 146). In this respect, PDGF and its alpha-receptor are noteworthy candidates for inducing tumorigenic transformation, as PDGF may induce transformation *in vitro* and *in vivo* (147, 148) and SVZ stem cells express the PDGF receptor alpha (148). However, recent evidence showed that adult stem cells are highly resistant to malignant transformation *in vitro* (149).

Materials and Methods

List of suppliers

Abcam (Cambridge, UK), Amersham (Freiburg, Germany), Amresco (Solon, OH, USA), Applera (Darmstadt, Germany), Axon Instruments (Foster City, CA, USA), Axxora (Lörrach, Germany), Biochrom (Berlin, Germany), Biomol (Hamburg, Germany), Calbiochem (Darmstadt, Germany), Cambrex (Rockland, USA), Covance (Berkeley, CA, USA), Dako (Glostup, Denmark), Dianova (Hamburg, Germany), Garner (Claremont, CA, USA), Invitrogen (Karlsruhe, Germany), Menzel (Braunschweig, Germany), Merck (Darmstadt, Germany), Microm (Heidelberg, Germany), Millipore (Chandlers Ford, UK), New England Biolabs (Frankfurt/Main, Germany), Olympus (Hamburg, Germany), Operon (Cologne, Germany), PAA (Cölbe, Germany), Promega (Mannheim, Germany), Qiagen (Hilden, Germany), R&D Systems (Minneapolis, USA), Roche (Mannheim, Germany), Santa-Cruz Biotechnology (Heidelberg, Germany), Sigma-Aldrich (Schnelldorf, Germany), Soft Imaging Systems (Münster, Germany), Swant (Bellinzona, Switzerland), Tebu-Bio (Offenbach, Germany), Tecan (Crailsheim, Germany), Uptima (Montelucon, France), Ventana (Strasbourg, France), Zeiss MicroImaging (Göttingen, Germany)

Materials

Preparation of reagents

BDNF

5 µg BDNF (R&D Systems) were dissolved in 0.5 ml PBS containing 0.1 % (w/v) BSA (Amresco) and used at a final concentration of 10 ng/ml.

BMP2

10 µg recombinant human BMP2 (R&D Systems) were dissolved in 200 µl of a sterile filtered solution containing 4 mM HCl (Sigma) and 0.1 % (w/v) BSA in aqua dest. Aliquots were stored for up to a year at -20°C. Final concentration was 50 ng/ml.

Dibutyryl cAMP

25 mg dibutyryl cyclic AMP (Sigma) were dissolved in 980 µl N5 medium (stock concentration 50 mM) and used at concentrations of 0.1 mM to 0.5 mM.

EGF and FGF2

200 µg recombinant human EGF (R&D Systems), 200 µg recombinant human FGF2 (R&D Systems) and 20 mg bovine heparin (Sigma) were dissolved in 10 ml N2 medium and added to cell cultures at a final concentration of 20 ng/ml (EGF and FGF2).

FGF-8

5 µg of recombinant human FGF8 (Tebu-Bio) were reconstituted in 50 µl of 10 mM Tris buffer (pH 8.0) and used at 100 ng/ml.

Forskolin

Forskolin (Sigma) was dissolved to a 5 mM stock solution in DMSO and used at a final concentration of 5 µM.

IBMX

100 mg IBMX (Sigma) were dissolved in 900 µl DMSO to obtain a 500 mM stock solution. IBMX was used at 0.5 mM.

LIF

10 µg recombinant human LIF (Millipore) were dissolved in 10 ml N5 medium and added to cell cultures at a final concentration of 10 ng/ml.

Histone deacetylase inhibitor stock solutions

M344 (Calbiochem), MS275 (Calbiochem) and SAHA (Axxora) each were dissolved in DMSO (Sigma) at a concentration of 10 mM. VPA (Sigma) was dissolved in sterile aqua dest at a concentration of 1 M. For final concentrations of histone deacetylase inhibitors, see text.

NGF

100 µg NGF (2.5 S isoform, Sigma) were dissolved in 40 ml PBS containing 0.1 % (w/v) BSA and stored at -20°C for up to a year. Final concentration was 25 ng/ml.

PPD

10 mg papain (Roche), 100 mg neutral protease (Dispase II, Roche) and 10 mg DNase I (Roche) were dissolved at room temperature in 100 ml HBSS without Ca^{2+} or Mg^{2+} (Invitrogen). After complete dissolution of reagents, $MgSO_4$ (Roth) was added to a final concentration of 12.4 mM. The solution was filter sterilized and stored at -20°C for up to three months.

Retinoic acid

A 0.1 M stock solution was freshly prepared before use by dissolving 50 mg all-trans retinoic acid (Sigma) in 1.67 ml DMSO. Retinoic acid was used at concentrations of 0.5 – 1 µM.

Shh

25 µg of recominant human sonic hedgehog peptide (R&D Systems) were dissolved in 500 µl PBS and stored at -20°C. Shh was used at 500 ng/ml.

Immunostaining solutions

Fish skin gelatine buffer (FSB)

Fish skin gelatine buffer consisted of 0.2 % (v/v) teleostean gelatine (Sigma), 1 % (w/v) BSA and 0.02 % (w/v) sodium azide (Merck) in TBS (prepared by dissolving 6.61 g Tris/HCl, 0.97 g Tris Base and 8.77 g NaCl in 1 L aqua dest). For intracellular antigen staining, 0.1 % Triton-X 100 (Sigma) was added.

Basic cell culture media

Unless stated otherwise, all media were supplemented with 1% penicillin-streptomycin solution (Sigma) and 2 mM L-glutamine (Invitrogen).

$D10^+$	DMEM (Biochrom), 10 % FCS (Biochrom)
N2	DMEM/Ham´s F-12 (1:1, Invitrogen), 5 mg/l insulin (Sigma), 50 nM progesterone (Sigma), 250 µM putrescine (Sigma), 72.28 nM sodium selenite (Sigma), 3.25 µM apo-transferrin (Millipore)
N5	N2 supplemented with 5 % (v/v) FCS and 35 µg/ml bovine pituitary extract (Invitrogen).

List of antibodies

Primary antibodies

Antigen	Host	Clonality	Dilution	Supplier	Comments
A2B5	mouse	monoclonal	1:250	Millipore	
CD133(human)	mouse	monoclonal	1:500	Gift of Dr. D. Corbeil, Dresden	
CD133(rodent)	rabbit	polyclonal	1:2,000	Gift of Dr. D. Corbeil, Dresden	clone K8 (150, 151)
CNPase	mouse	monoclonal	1:200	Millipore	
Dlx2	rabbit	polyclonal	1:400	Abcam	
Doublecortin	goat	polyclonal	1:200	SantaCruz	
GAD65/67	rabbit	polyclonal	1:200	Millipore	
GFAP	mouse	monoclonal	1:600	Millipore	
GFAP	rabbit	polyclonal	1:600	Dako	
Glutamine synthetase	rabbit	polyclonal	1:100	Abcam	
Map2a-c	mouse	monoclonal	1:25	Gift of Dr. B. Riederer, Lausanne	
Map2ab	mouse	monoclonal	1:250	Sigma	
Musashi-1	rabbit	polyclonal	1:200	Millipore	
Nestin (human)	rabbit	polyclonal	1:4,000	Millipore	
Nestin (rodent)	mouse	monoclonal	1:400	Millipore	clone Rat-401 (152)
Olig2	mouse	monoclonal	1:200	Millipore	
p21	mouse	monoclonal	1:30	Dako	
p53	mouse	monoclonal	1:100	Millipore	
PDGFR	mouse	monoclonal	1:50	Millipore	
PSA-NCAM	mouse	monoclonal	1:200	Millipore	
S100β	rabbit	polyclonal	1:1,000	Swant	
Tubulin βIII	mouse	monoclonal	1:500	Promega	
Tubulin βIII	rabbit	polyclonal	1:500	Covance	
Tyrosine hydroxylase	rabbit	polyclonal	1:200	Millipore	
Vimentin	mouse	monoclonal	1:800	Dako	
Vimentin	rabbit	polyclonal	1:500	Biomol	
vGlut1	rabbit	polyclonal	1:500	Sigma	

Secondary antibodies

Host	Antigen	Fluorochrome	Dilution	Supplier
Goat	anti-mouse	Alexa 488	1:200	Invitrogen
Goat	anti-mouse	Cy3	1:300	Dianova
Goat	anti-rabbit	Cy2	1:300	Dianova
Goat	anti-rabbit	Cy3	1:300	Dianova
Donkey	anti-goat	Alexa 488	1:200	Invitrogen

Animals

Wistar rats

Postnatal and adult Wistar rats were obtained both from Charles River Wiga (Sulzfeld, Germany) and from the Franz-Penzoldt-Zentrum (animal housing and breeding facility of the University Erlangen). Animal experiments were approved by the local government (Regierung von Mittelfranken).

C57/Bl6 mice

Postnatal C57/Bl6 mice were obtained from Charles River Wiga.

Human material

Human tissue was obtained from hippocampal resections performed for epilepsy surgery at the University Hospital Erlangen. Control tissue was obtained from hippocampi of autopsy specimens. Informed consent of patients or relatives was obtained in both cases. This study was approved by the local ethics committee of the University of Erlangen.

List of primers

Unless stated otherwise, all primers were ordered from Operon.

Gene Name	Accession Nr	Forward sequence	Reverse sequence
Atf5	NM_030693	QT00246148*	
Btg2	NM_007570	QT00153216*	
Btg3	NM_009770	QT00107548*	
CyclinD1(Ccnd1)	NM_007631	QT00154595*	
CyclinD2 (Ccnd2)	NM_009829	QT00170618*	
CyclinD3 (Ccnd2)	NM_007632	QT00146615*	
Neurod1	NM_010894	QT00251265*	
Olig1	NM_016968	QT01041082*	
Olig2	NM_016967	QT01041089*	
β Actin (Actb)	NM_031144.2	gagaagagctatgagctgcc	tccatacccaggaaggaagg
Dlx1	XM_230987.4	ctacgtcaactcggtcagca	ccgttaaagcgcacttctcc
Dlx2	XM_230986.4	gcagctactacaccaaccag	gtcgggctcgttgttgactg
Dlx5	NM_012943.1	caggaatcgccaactttgcc	gcggtaagaagcagaggtag
Egfr	NM_031507.1	tggagagaatccctttggag	ggttccatagttggacagga
Gapdh	NM_017008.3	ggctggcattgctctcaatg	catgtaggccatgaggtcca
Gfap	NM_017009.1	acatcgtggtaaagacggtg	cctgttagttggagatgcag
Musashi-1 (Msi1)	NM_148890.1	ggaaaggtggatgatgccat	catggaagtggatctcacac
Nestin (Nes)	NM_012987.1	tggaacagagattggaaggc	gactaagggacatcttgagg
Olig2 (predicted)	XM_221668.4	cggtggcttcaagtcatctt	gggctcagtcatctgcttct
p53 (Tp53)	NM_030989.1	cctccagatgatattctgcc	ggccttctaacaactctgca
p21 (Cdkn1a)	NM_080782.3	acggtggaactttgacttcg	gacccagggctcaggtagat
Pdgf-A	NM_012801	atgccttggagacaaacctg	acttctcttcctgcgaatgg
Pdgf-B	XM_343293	acacctcaaactcgggtgac	tcagtgccttcttgtcatgg
Pdgfrα	XM_214030.3	ccattctggagcttgtggat	atccactcgatgttcggaag
Pten	NM_031606.1	gttcttcataccaggaccag	ccgcacgctctatactacaa
Sox2	XM_985079.1	agatgcacaactcggagatc	gagtaggacatgctgtaggt

* these primers are part of the Quantitect Assays and were ordered from Qiagen

Methods

Preparation and culture of neural precursor cells

Animal tissue

For the isolation of rodent neural precursor cells, postnatal animals were anesthetized, decapitated, the brains removed and placed into ice-cold PBS (Biochrom). Afterwards, brains were dissected and the subventricular zone isolated. SVZ tissue was pooled and enzymatically dissociated using PPD solution (5 ml per 5 brains) for 30 minutes at room temperature. The resulting cell suspension was centrifuged at 150 x g, washed twice in PBS and resuspended in N2 medium. Cells were plated at a density of 50,000 to 100,000 cells per cm^2.

EF was added to the culture medium every third day and medium exchange was performed once a week. Neurospheres formed within 3 DIC and were passaged when mean sphere size reached a diameter of about 200 – 500 µm (normally every three days). For passaging, neurospheres were collected by centrifugation, resuspended in 100 µl Accutase solution (PAA) and incubated for 15 minutes at 37°C. The cell suspension was resuspended using a pipette and pelleted by centrifugation followed by washing in PBS. Thereafter, cells were plated at a density of 50,000 to 100,000 cells per cm^2.

Human tissue

For the isolation of adult human precursor cells, the dentate gyrus was prepared from hippocampal tissue specimens.

The tissue was dissociated mechanically using scalpels followed by enzymatic digestion in PPD solution (supplemented with penicillin-streptomycin solution (Sigma) and Fungizone (Invitrogen) for autopsy specimens) for 30 minutes at room temperature. The cell suspension was washed once in PBS and centrifuged in a 0.9 M sucrose/0.5x HBSS solution gradient. The supernatant was removed and the pellet resuspended in PBS, centrifuged at 150 x g and finally resuspended in N5 medium and plated at a density of 20,000 cells per cm^2 onto poly-L-ornithine/laminin (Sigma) coated plates.

EF and LIF were added to the cell culture medium every third day and medium exchange was performed once a week. For passaging, cells were grown to confluence, mechanically removed from the culture surface, pelleted by centrifugation and resuspended in 1 ml Accutase solution (PAA). Following digestion for 15 minutes at 37°C, the cells were collected by centrifugation, counted in a Neubauer chamber and split 1:2.

Cell viability assay (MTT assay)

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay (153) was used to assess cell viability after treatment with different compounds (see Results). Cells were plated in 96-well culture plates at a final concentration of 2,000 cells per well. One hour after plating, cells were treated with different concentrations of the respective compounds. 96 hours after incubation, 0.5 mg/ml MTT (Sigma) dissolved in medium was applied for 2 hours. Subsequently, the medium was discarded and each well was incubated with 100 µl of a 2-propanol stock solution containing 165 µl HCl per 50 ml 2-propanol (Merck). The optical density of each well was

determined using a microplate reader (Tecan) set to a wavelength of 550 nm (wavelength correction set to 690 nm).

Neurosphere assay

For clonal expansion analysis, cells were plated at a density of 10,000 cells per cm^2 in N5 medium containing 1 % (w/v) methylcellulose (4,000 cP, Sigma) into agarose-coated 6-well plates. EF was added every third day. After 21 DIC, sphere numbers were counted using phase contrast microscopy in 10 visual fields of 0.6 mm^2 each.

Differentiation assay

For analysis of neural precursor cell differentiation, 50,000 cells were plated onto coverslips (18 mm diameter, Microm) coated with poly-L-ornithine and laminin. Differentiation conditions for rodent precursor cells were N2 medium without growth factors (EF) and supplemented with 10% FCS. Differentiation conditions for human precursor cells were N2 medium supplemented with bovine pituitary extract and 1% FCS for 48 hours.

Fluorescence immunocytochemistry

For immunocytochemistry, cells were fixed in 4 % paraformaldehyde (Sigma) for 30 minutes at room temperature followed by three washes in PBS (Biochrom). After fixation, cells were blocked using FSB (with Triton-X 100 (Sigma) for

intracellular antigen staining) for 2 hours at room temperature. Primary antibodies were diluted in FSB (with Triton-X 100 added where appropriate) and incubated over night at 4°C. Following three washes with PBS for 10 minutes, incubation with secondary antibodies (diluted in FSB, with Triton-X 100 added where appropriate) was performed for four hours at room temperature. Nuclei were counterstained with Hoechst 33342 (Sigma) diluted in FSB for 15 minutes at room temperature followed by washing in FSB and PBS for 10 minutes each. Coverslips were then mounted using aqueous mounting medium with anti-fade (Sigma).

Immunohistochemistry

For immunostaining of 4 % paraformaldehyde-fixed, paraffin-embedded tissue, 4 µm sections were cut with a microtome (Microm), and mounted on positively charged slides (Superfrost+, Menzel). The slides were air dried in an incubator at 37°C over night. Immunohistochemical reactions were performed using an automated staining system (Ventana) and the streptavidin-biotin method using 3,3'-diaminobenzidine (Ventana) as chromogen and hematoxylin counterstaining (154).

Side population analysis and cell sorting

For side population analysis, cells were incubated with 5 µg/ml Hoechst 33342 (Sigma) for 90 minutes at 37°C in the dark, followed by washing in PBS (Biochrom). Controls consisted of Hoechst 33342 staining in the presence of 100 µM Verapamil (Sigma). Cells were analyzed with a MoFlo

Flurorescence-Activated Cell Sorter (FACS; Dako Cytomation).

For single-cell experiments, cultured neurospheres were dissociated using Accutase (PAA), pelleted and resuspended in PBS supplemented with 2 mM EDTA (Sigma). Individual cells were sorted into the wells of a 96-well cell culture plate using a MoFlo FACS (Dako Cytomation). Sorting efficiency was confirmed microscopically and only wells containing single cells analyzed further.

Protein isolation, SDS-PAGE and western blot

For protein extraction and western blot of acetylated histones, murine neurosphere-derived cells were differentiated on a laminin/poly-ornithine coated surface in the presence of 10 % FCS (Biochrom) and either HDAC inhibitors or solvent control. Cells were collected, pelleted by centrifugation, washed three times in PBS (Biochrom) and suspended in lysis buffer (containing 10 mM HEPES, pH 7.9 (Biochrom); 1.5 mM $MgCl_2$ (Merck); 10 mM KCl (Merck); 0.5 mM DTT (Sigma); 1.5 mM PMSF (Sigma)). Sulfuric acid (Merck) was added to a final concentration of 0.2 mM. After incubation on ice, acid-insoluble proteins were centrifuged and the supernatant was dialyzed against 0.1 M acetic acid (Merck) and H_2O. Protein concentrations were determined via bicinchoninic acid assay (BCA, Uptima). Ten micrograms of protein extract were separated on a 12 % sodium dodecylsulfate polyacrylamide gel, followed by electroblotting onto nitrocellulose. Mouse anti β-actin (1:10,000; Sigma) and mouse anti-acetyl histone H3 (Lys 9; 1:450; Millipore) antibodies were used for protein detection

and visualized with ECL kit (Amersham) according to the manufacturer's protocol.

RNA isolation and quantitative real-time PCR

Extraction of RNA was performed as described (155). For cDNA synthesis, the SuperScript II first strand synthesis kit (Invitrogen) was used according to the manufacturer's instructions. PCR reactions were performed in a total volume of 10 µl using 20 ng of total cDNA in a PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen), 5 pmol of each primer, 200 µM of each dNTP and 0.25 U Taq Polymerase (Qiagen). The cycling conditions were 40 cycles of denaturation at 94°C for 35 s, annealing at 55°C for 40 s and extension at 72°C for 40 s. Quantitative real-time PCR reactions were performed using the Power SYBR green PCR master mix (Applera) according to the manufacturer's instructions on an Applied Biosystems 7500 Real-Time PCR System. Cycling conditions for real-time PCR were 40 cycles of denaturation at 95°C for 30 s, followed by annealing at 60°C for 60 s and extension at 72°C for 30 s.

In some experiments, quantification of mRNA levels was performed using the one step QuantiTect SYBR Green RT-PCR Kit and validated QuantiTect Primer Assays (Qiagen). In these cases, SPCs from the SVZ of postnatal day six C57/Bl6 mice were cultured and differentiated according to the above-mentioned protocol. During differentiation, cells were treated with iHDAC in optimized concentrations and exposition times (see Results, p. 45f.). Amplicons have been designed to span exon borders to exclude false positive detection of genomic contaminations. Each 20 µl RT-PCR mix contained 10 ng total RNA, 2 µl of the QuantiTect Primer Assay, 10 µl 2 x

QuantiTect SYBR Green RT-Master Mix and 0.2 µl QuantiTect RT Mix. Cycling conditions were 50°C for 30 min, 95°C for 15 min and 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 35 sec. Real-time RT-PCR was conducted four times for each gene of every RNA sample. The comparative method of relative quantification ($2^{-\Delta\Delta Ct}$) was used to calculate the relative expression levels of each target gene (normalized to GAPDH) and compared to the non-treated control samples. RT-PCR specificity of each reaction was verified by melting curve analysis.

β-Galactosidase staining

Immunohistochemical staining for the senescence-associated marker β-Galactosidase (156) was performed using the Senescence beta-Galactosidase staining kit (New England Biolabs) according to the manufacturer's instructions. Briefly, cells and tissues were fixed with a combination of formaldehyde and glutaraldehyde, and the activity of β-Galactosidase at pH 6.0 was determined by conversion of X-gal into a blue substrate.

Animal transplantation

50,000 cells were transplanted into the brains of 300g Wistar rats at the coordinates Bregma + 1.20 mm, lateral – 2.5 mm, vertical – 4.5 mm (157) in a total volume of 5 µl. All animals were handled according to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the German Law on the Protection of Animals. Neurological deficits were scored every day [grade 0: normal; grade 1: tail

weakness or tail paralysis; grade 2: hind leg paraparesis or hemiparesis; grade 3: hind leg paralysis or hemiparalysis; grade 4: complete paralysis (tetraplegia), moribund stage or death]. Tetraplegic animals were killed. Animals were deeply anaesthetized, perfused using 4 % paraformaldehyde, the brains removed and embedded in paraffin for immunohistochemistry.

Chromosome preparation

Cells were grown as monolayer in 5 ml of N2/10% FCS (see above), treated with 100 µl Colcemid (10 µg/ml) (Roche) for 120 minutes at 37° C. The cells were detached by treatment with trypsin-EDTA (Biochrom) and centrifuged. The cell pellet was suspended in hypotonic solution (75 mM KCl) for 20 minutes at 37°C, centrifuged, and fixed in methanol and acetic acid. Metaphase spreads were prepared on slides, dried, and Giemsa stained after trypsin pretreatment.

Microscopical evaluation

Qualitative and quantitative analysis of immuno-cytochemically identified cell types was performed using high power optical fields digitized using a BX51 microscope (Olympus) equipped with a F View II CCD camera (Soft Imaging Systems) or an IX70 microscope (Olympus) equipped with a Color View II camera (Soft Imaging Systems) and respective imaging software (analySIS, Soft Imaging Systems). Nuclear counterstaining (Hoechst 33342) was quantified in all experiments and used as reference value to assess the percentage of neuronal and glial subpopulations. Statistical

significance was calculated using Student´s t-Test (GraphPad Prism 4.02, GraphPad Software, USA).

siRNA transfection

siRNA molecules targeted against the PDGFRα sequence (XM_001067574) were obtained from Qiagen (sequences given below). To test cell growth reduction, R2303 cells were seeded at 5,000 cells/well in 96-well plates and transfected with the PDGFRα siRNAs at concentrations of 5 nM and 10 nM, respectively, using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Controls consisted of cells incubated with Lipofectamine only and cells transfected with oligonucleotides with no sequence similarity to any known rodent gene. Cell viability was analyzed 7 days after transfection using the MTT assay as described above.

siRNA Name	**Sense Sequence**	**Antisense Sequence**
Pdgfra_1	r(CGCCCAUUAUACCAUUAUA)dTdT	r(UAUAAUGGUAUAAUGGCCG)dCdT
Pdgfra_2	r(AGCAACGUCUCGAAUAUUA)dTdT	r(UAAUAUUCGAGACGUUGCU)dGdG
Pdgfra_3	r(CCAGCGAGUUUAACGUUUA)dTdT	r(UAAACGUUAAACUCGCUGG)dTdC

Previous work

Dose – effect characterization of histone deacetylase inhibitors on growth inhibition of tumor cells

F98 rat malignant glioma cells were treated with different concentrations of iHDAC CBHA, M344, MS-275, phenylbutyrate, SAHA, SBHA and VPA. The range of concentrations covered three orders of magnitude. In order to test the potency of these compounds to reduce cell growth, the MTT cell viability assay was performed (158, 159). For all substances, the IC_{50} and IC_{90} based on cell growth reduction were calculated and used as lead concentrations for following experiments (Table 1 and Fig. 8). It is interesting to note that IC_{50} values were similar between compounds of same HDAC inhibitor classes, i.e. benzamides and hydroxamic acids (Fig. 8). Another intriguing observation was that there are two subgroups of hydroxamic acids with different efficacy. SAHA and M344 differ from CBHA and SBHA in their efficacy by more than one decade. The most potent substances of each class of HDAC inhibitors were selected for further experiments: the hydroxamic acids SAHA and M344, MS-275 as benzamide and VPA as aliphatic acid.

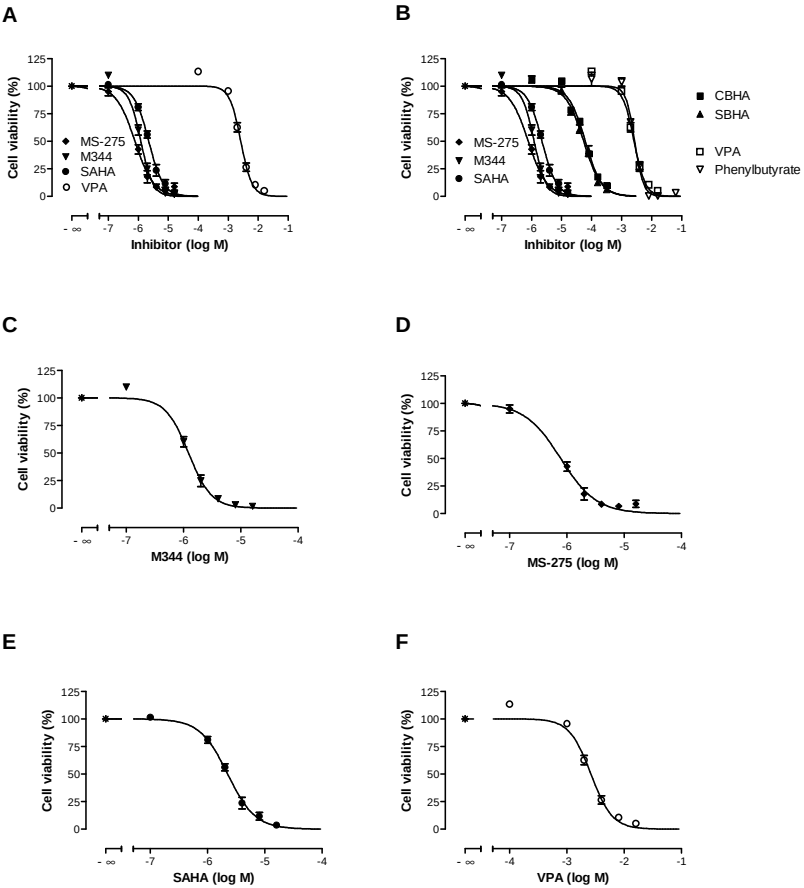


Figure 8: Concentration-effect curves of different HDAC inhibitors on F98 cell viability. Ordinate: Cell viability expressed as percentage of control values in the absence of an inhibitor. Abscissa: log concentration of the tested compounds. Curves were generated by logistic nonlinear regression analysis. Given are mean values ± SEM (n=8). **A**, all tested iHDAC. **B**, iHDAC used in the following experiments. **C-F**, single curves for indicated HDAC inhibitors.

Substance	pIC_{50}	IC_{50}	n_H	pIC_{90}	IC_{90}
MS-275	6.11 ± 0.01	0.78 µM	-1.39 ± 0.05	5.43	3.72 µM
M344	5.92 ± 0.01	1.20 µM	-2.18 ± 0.10	5.48	3.31 µM
SAHA	5.65 ± 0.01	2.24 µM	-1.81 ± 0.02	5.12	7.59 µM
CBHA	4.21 ± 0.01	61.66 µM	-1.58 ± 0.06	3.60	251.19 µM
SBHA	4.27 ± 0.01	53.37 µM	-1.48 ± 0.04	3.62	239.88 µM
VPA	2.58 ± 0.01	2.63 mM	-2.33 ± 0.12	2.17	6.76 mM
Phenylbutyrate	2.56 ± 0.01	2.75 mM	-2.99 ± 0.14	2.24	5.75 mM

Table 1: Parameters describing the inhibition of cell viability by the indicated compounds in F98 cells (cf. Fig. 5 A). pIC_{50}, negative decade logarithm of IC_{50}. IC_{50}, concentration of the tested compound inducing a 50% inhibition of cell viability compared to controls. n_H, curve slope. All curve slopes were different from unity (F-Test, $p>0.05$). Therefore, pIC_{50} were obtained from curves with variable curve slopes. pIC_{90}, negative decade logarithm of IC_{90}. IC_{90}, the concentration which is necessary to drop cell viability by 90% compared to controls. Given are mean values ± SEM of 8-12 experiments as derived by nonlinear regression analysis applying a four parameter logistic equation (in cooperation with PD Dr. C. Tränkle, Pharmaceutical Dept., University of Bonn).

HDAC inhibitors induce differentiation in a malignant glioma cell line

Treatment of F98 cells with HDAC inhibitors altered cell morphology, which was visible 24 hours after treatment and reached a maximum effect after 48 hours. Prolonged treatment protocols induced excessive cell death (data not shown, but see (160)). Altered morphology of F98 cells was characterized by outgrowth of long, thin processes at two opposite poles of the cell body (bipolar phenotype) that further branched in their distant segments (Fig. 9). To assess the extent of process outgrowth, which is compatible with advanced differentiation, the length of whole cells was measured and is summarized in table 2. No significant differences in cell length between untreated and vehicle treated cells were found, while tumor cell length grew with increasing HDAC inhibitor concentration.

MS-275	**Co**	**DMSO**	**1 µM**	**2 µM**	**4 µM**	**8 µM**
Cell length (µm)	67.93 ± 12.50	69.65 ± 11.83	97.66 ± 20.01	123.00 ± 24.20	137.19 ± 35.22	141.42 ± 48.29
M344	**Co**	**DMSO**	**1 µM**	**2 µM**	**4 µM**	**8 µM**
Cell length (µm)	74.41 ± 16.20	64.67 ± 11.47	99.81 ± 24.25	101.59 ± 20.64	128.00 ± 28.14	145.07 ± 33.73
SAHA	**Co**	**DMSO**	**1 µM**	**2 µM**	**4 µM**	**8 µM**
Cell length (µm)	60.90 ± 13.37	60.77 ± 15.14	82.97 ± 17.10	105.35 ± 16.89	139.91 ± 27.98	158.74 ± 48.75
VPA	**Co**	**0.1 mM**	**0.5 mM**	**1 mM**	**2 mM**	**10 mM**
Cell length (µm)	59.64 ± 13.15	74.27 ± 17.92	90.88 ± 18.37	106.51 ± 17.18	125.91 ± 18.16	198.13 ± 61.46

Table 2: Effects of different HDAC inhibitor concentrations on the differentiation of F98 glioma cells (n=40 each). Given are mean cell length ± SD. Increased tumor cell length was statistically significant ($p<0.01$; Mann-Whitney U-Test) between controls and HDAC inhibitor treated cells. Co, untreated controls.

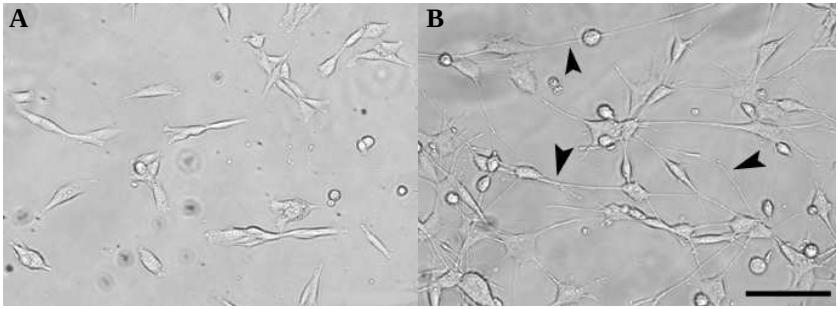


Figure 9: Cellular morphology of control and HDAC inhibitor treated F98 cells. A, untreated F98 cells 48 hours after plating. **B**, F98 cells treated with 10 mM VPA for 48 hours. Arrowheads indicate process outgrowth following treatment. Scale bar, 100 µm.

Results

Preparation of rodent neural precursor cells and expansion in culture

Postnatal rat SVZ precursor cells (SPCs) were expanded at a density of 20,000 cells per cm^2 that has been reported to achieve clonal growth (139, 161). The cells were expanded in suspension culture in defined medium supplemented with growth factors (EGF and FGF2), which resulted in formation of free-floating spheres. To establish stemness of expanded cells, the Neurosphere Assay (162) was performed from freshly prepared cells as well as from spheres that have been cultured for several passages. Both populations formed clonal neurospheres that proved multipotent upon differentiation (Fig. 10). Furthermore, single cells were sorted individually into the wells of 96-well culture plates using a MoFlo cell sorter and assessed for clonal growth of neurospheres. The efficiency of single cells to form multipotent neurospheres was 22 cells out of 288 (7.6 %).

Multipotency was assessed by immunostaining for the following markers: O4 surface glycoprotein (163) and CNPase (164) for oligodendrocytes, GFAP (165) and S100β (166) for astrocytes and Map2 (120) and tubulin βIII (167) for neurons. All spheres stained for markers of neuronal as well as glial lineages, thus confirming the multipotency of the original sphere–forming precursor cell (Fig. 10). The cultured neurospheres could be continuously passaged and were multipotent in each passage. Only neurospheres after passage 2 were used in subsequent experiments.

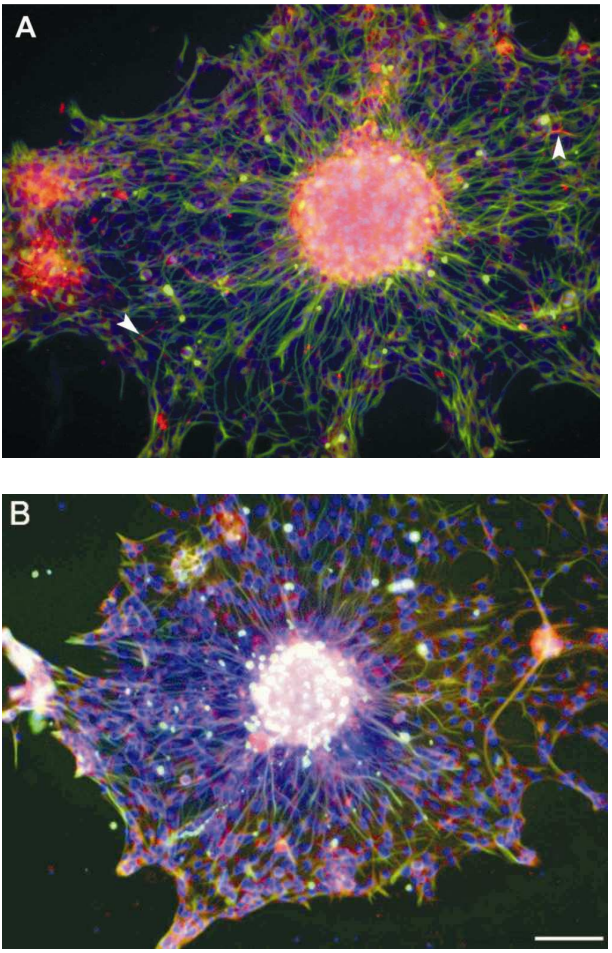


Figure 10: Immunostaining of multipotent neurospheres. Cells extend processes and migrate out of a central plated sphere. **A**, Multipotency for cells of neuronal and glial lineages is established by immunostaining for astrocytes (identified by GFAP expression, green) and neurons (stained for βIII tubulin, red). Nuclei are labelled by Hoechst (blue). Individual neuronal processes can be distinguished (arrowheads). **B**, Cells of the glial lineage are further distinguished by expression of GFAP (green) or the oligodendrocyte marker O4 (red). Nuclei are labelled by Hoechst (blue). Scale bar, 100 µm.

Differentiation of rodent neural precursor cells in vitro

For *in vitro* differentiation, rodent neurospheres in passage 2 were plated onto laminin/poly-ornithine coated cover slips and placed into N2 medium containing 10 % FCS without growth factors. The cells were allowed to differentiate for seven days, subsequently fixed and stained for markers of neuronal and glial lineages (Fig. 11). Using βIII tubulin, GFAP and CNPase staining, a robust and reproducible ratio of approx. 5 % neurons (5.4 ± 0.5 %), 22 % oligodendrocytes (21.5 ± 1.6 %) and 45 % astrocytes (44.6 ± 1.3 %) was found (n=3 for each marker tested and repeated in at least two independent settings). A subpopulation of cells remained that could not be stained for markers of differentiated cells. This population was calculated to amount to approx. 25 % (Fig. 13). For absolute cell numbers, see table 3.

Exp. #	Total cells	# neurons	# astrocytes	# oligo-dendro-cytes	% neurons (± SEM)	% astrocytes (± SEM)	% oligodendro-cytes (± SEM)
1	3807	n.d.	1698	846	n.a.	44.6 ± 1.3	22.2 ± 1.6
2	2547	139	911	n.d.	5.4 ± 0.4	34.7 ± 2.2	n.a.

Table 3: Differentiated progenies from neurosphere-derived cells. Given are data obtained from two representative experiments. SEM, standard error of the mean.

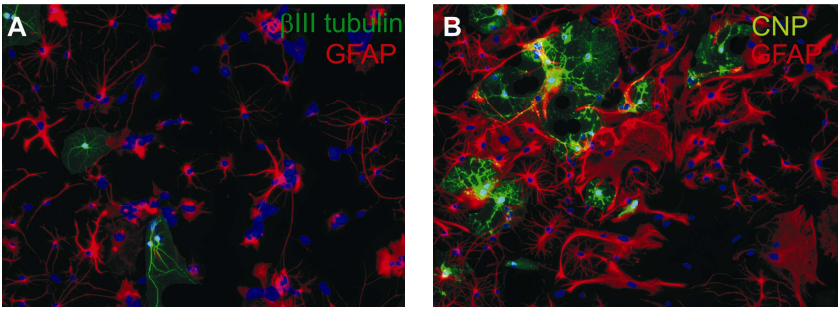


Figure 11: Differentiation of neurosphere derived progeny. A, Neurons and astrocytes are identified by expression of bIII tubulin or GFAP. Numbers of neurons are very low. **B**, Oligodendrocytes are characterized by CNPase staining. Different types of astrocytes can be identified according to their morphology (**A** and **B**). Magnification 100x.

Histone deacetylase inhibitors increase the amount of neuronal differentiation from adult forebrain precursor cells

To test the effect of HDAC inhibitors on SPC differentiation, neurosphere-derived cells were differentiated for seven days by growth factor withdrawal and serum addition. One hour after plating, HDAC inhibitors VPA, SAHA, MS-275 and M344 or DMSO vehicle were added to the cultures for the entire experiment. The potency of HDAC inhibitors VPA and SAHA to affect histone acetylation was confirmed by immunoblotting for Lys-9 acetylated histone H3 (Fig. 12).

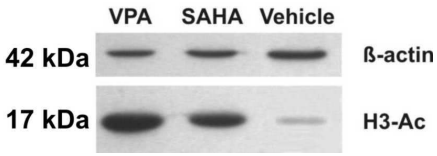


Figure 12: Western Blot for acetylated Histone H3. HDAC inhibition by VPA or SAHA for 48 hours induced histone acetylation compared to vehicle treated control experiments. Beta-actin was used as loading control.

Numbers of differentiated neurons were assessed by double-immunostaining for βIII tubulin and GFAP. To exclude potential cell culture artifacts, βIII tubulin/GFAP double-labeled cells were not counted into either class (neuronal or glial). Total as well as immunohistochemically identified cell numbers were counted in 10 randomly placed visual fields (0.6 mm^2 each). Each experiment was performed in triplicate and the number of total cells counted for all three experiments was at least 1,000 cells. All tested compounds were able to significantly increase numbers of neurons in differentiating SPCs (Table 4).

Concentration-effect evaluation

To examine whether the iHDAC-mediated effect was concentration-dependent, differentiating neurosphere-derived cells were incubated for seven days with different concentrations of VPA (ranging from 0.2 mM to 2 mM), MS-275, SAHA or M344 (0.1 µM to 2 µM, respectively). All compounds increased neuronal numbers with peak numbers at following concentrations: 0.5 µM SAHA, 1 mM VPA, 1 µM MS-275 and 1 µM M344, respectively (Table 4, Fig. 13). In contrast, reduced cell numbers were observed at concentrations of >1 µM of SAHA, M344 and MS-275.

After analysis of iHDAC concentration effects, it was analyzed whether short-term exposure to iHDAC was sufficient to induce neuronal differentiation. HDAC inhibitors VPA (1 mM), SAHA (0.5 µM), MS-275 (1 µM) and M344 (1 µM) were added to differentiating neurosphere-derived cells. Inhibitors were washed out and medium was exchanged after 24 hours and 48 hours, respectively. The most pronounced increase in neuronal differentiation was found after 24 hours of treatment with VPA, M344 and MS-275 and after 48 hours with SAHA (Table 4, Fig. 13). This increase was found to be more prominent than long-term exposure over seven days.

Substance	Conc.	Exposure time	# total cells	# neurons	# astrocytes	# oligo-dendrocytes	% neurons (± SEM)	% astrocytes (± SEM)	% oligo-dendrocytes (± SEM)
M344	0.2 µM	7 days	1632	156	387	n.d.	9.6 ± 0.6	23.4 ± 1.0	n.a.
	0.5 µM	7 days	1626	165	474	n.d.	10.1 ± 0.6	28.8 ± 1.2	n.a.
	1 µM	7 days	3441	423	1383	n.d.	12.3 ± 1.2	46.3 ± 2.4	n.a.
	2 µM	7 days	317	31	72	n.d.	9.8 ± 2.0	23.2 ± 1.8	n.a.
	1 µM	24 hours	3199	609	1671	n.d.	19.0 ± 0.8	48.5 ± 1.3	n.a.
	1 µM	48 hours	2561	438	1257	n.d.	17.1 ± 0.7	49.0 ± 2.1	n.a.
	1 µM	24 hours	1390	n.d.	750	141	n.a.	37.1 ± 2.2	10.1 ± 0.7
MS-275	0.2 µM	7 days	9783	657	2508	n.d	6.7 ± 0.3	25.4 ± 0.9	n.a.
	0.5 µM	7 days	5266	459	1380	n.d	8.7 ± 0.6	26.3 ±0.7	n.a.
	1 µM	7 days	4017	589	1537	n.d	14.7 ± 1.1	44.6 ± 2.3	n.a.
	2 µM	7 days	3837	309	1155	n.d	8.1 ± 0.6	32.5 ± 1.3	n.a.
	1 µM	24 hours	3711	648	1962	n.d.	17.5 ± 0.7	48.7 ± 0.9	n.a.
	1 µM	48 hours	3033	462	1587	n.d.	15.2 ± 0.7	53.1 ± 1.4	n.a.
	1 µM	24 hours	1995	n.d.	1146	228	n.a.	36.6 ± 1.9	11.4 ± 0.7
SAHA	0.1 µM	7days	1748	243	765	n.d	13.9 ± 1.1	46.1 ± 2.0	n.a.
	0.2 µM	7 days	2234	366	627	n.d	16.4 ± 0.9	29.4 ± 1.5	n.a.
	0.5 µM	7 days	2842	474	825	n.d	16.7 ± 1.1	29.9 ± 1.4	n.a.
	1 µM	7 days	4079	465	1890	n.d	11.4 ± 1.2	51.5 ± 2.4	n.a.
	0.5 µM	24 hours	2973	753	1317	n.d.	25.3 ± 1.0	45.6 ± 1.4	n.a.
	0.5 µM	48 hours	2636	717	1206	n.d.	27.2 ± 1.3	46.5 ± 1.2	n.a.
	0.5 µM	48 hours	1737	n.d.	792	213	n.a.	46.6 ± 1.5	12.3 ± 0.6
VPA	0.2 mM	7 days	1971	156	738	n.d	7.9 ± 0.5	38.5 ± 1.8	n.a.
	0.5 mM	7 days	2029	168	783	n.d	8.3 ± 0.4	39.3 ± 1.5	n.a.
	1 mM	7 days	5656	888	1632	n.d	15.7 ± 1.2	31.2 ± 1.9	n.a.
	2 mM	7 days	834	138	420	n.d	16.5 ± 1.1	50.1 ± 2.2	n.a.
	1 mM	24 hours	1366	264	522	n.d.	19.3 ± 1.4	44.7 ± 1.4	n.a.
	1 mM	48 hours	1773	291	762	n.d.	16.4 ± 1.0	44.6 ± 2.9	n.a.
	1 mM	24 hours	1680	n.d.	876	183	n.a.	41.5 ± 1.4	10.9 ± 0.6

Table 4: Cell numbers identified by fluorescence immunostaining after iHDAC treatment of neurosphere-derived cells. Every row represents one assay consisting of three independent experiments, each comprising of 10 random visual fields. SEM, standard error of the mean.

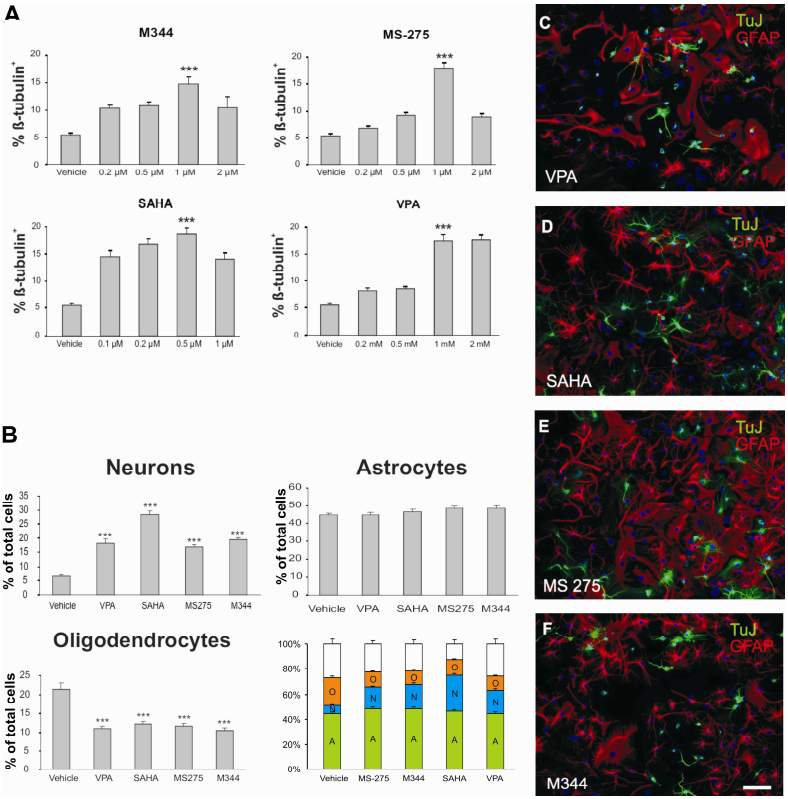


Figure 13: Effects of iHDAC treatment on differentiating neurosphere-derived cells. **A**, concentration-effect diagrams for different iHDACs. Given is the percentage of βIII tubulin positive cells from total cells as identified by Hoechst nuclear staining. Vehicle: DMSO. Asterisks indicate statistical significance (t-Test, $p<0.01$). **B**, Effects of iHDAC treatment in optimized concentrations and exposure times (see text) on distribution of subpopulations. Neurons were identified by βIII tubulin immunostaining, astrocytes by GFAP and oligodendrocytes by CNPase staining. Data are presented from different experiments (see text and Table 4). Asterisks indicate statistical significance over vehicle treated cells (t-Test, $p<0.01$). Calculation of total differentiated cells reveals a population (white bars) whose fate cannot be determined by above used markers. This population is unchanged for all iHDAC except SAHA. A, Astrocytes (green bars). N, Neurons (blue bars). O, Oligodendrocytes (orange bars). **C-F**, immunostaining of neurosphere-derived cells after iHDAC treatment in optimized concentrations and exposure times (see text) and a total of seven days of differentiation. Neurons are green (TuJ, βIII tubulin), astrocytes are red (GFAP) and nuclei are blue (Hoechst). Scale bar, 20 µm.

To analyze whether HDAC inhibitors influenced cell numbers of other lineages, a systematic double-immunofluorescence analysis was performed. Neurosphere-derived cells were differentiated for seven days and treated with HDAC inhibitors (using optimum concentrations and exposition times as determined from earlier experiments mentioned above) or vehicle and subsequently immunostained for either βIII-tubulin and GFAP or CNPase and GFAP. All compounds tested increased the number of differentiated neurons, while oligodendrocyte numbers decreased ($p<0.001$, t-Test). No significant changes in the astrocytic cell population characterized by GFAP immunoreactivity were observed (t-Test; Fig. 13 and Table 4). Calculation of total differentiation from different experiments showed that a population of cells (vehicle: 27.31 % ± 3.51 %; MS-275: 22.68 % ± 2.29 %; M344: 21.65 % ± 2.85 %; SAHA: 12.94 % ± 3.10 %; VPA: 26.06 % ± 3.39 %) did not stain for any of the markers used. This population was approximately equal for vehicle and iHDAC treated experiments with the exception of SAHA. In SAHA treated experiments, the unstained population amounted to half the size as in all other experiments.

Propidium iodide assay

A propidium iodide incorporation assay was performed to examine toxicity of the used compounds. Cell death of differentiating iHDAC or vehicle treated cells was examined by the incorporation of propidium iodide and assessed after 24, 48 and 72 hours, respectively. Numbers of propidium iodide positive cells were quantified and the ratio of propidium iodide positive cells versus total cells calculated. None of the second generation iHDAC induced cell death at low concentrations (0.5 µM – 1 µM). However, higher concentrations of second generation iHDAC (2 µM – 5 µM) as well as VPA at all concentrations (1 mM – 5 mM) prominently increased propidium iodide incorporation (Fig. 14), compatible with enhanced cell death (168).

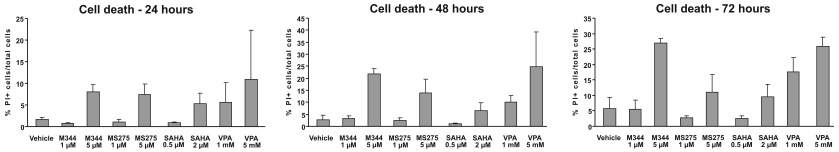


Figure 14: Cell death induced by iHDAC. Differentiating neurosphere-derived cells were treated with different concentrations of iHDAC for 24, 48 or 72 hours, respectively. Following iHDAC exposure, the amount of induced cell death was estimated by propidium iodide incorporation. Given are percentages of PI positive cells of the total cell population (as identified by light microscopy). While low concentrations of iHDAC do not increase cell death compared to vehicle treated cells, higher concentrations of iHDAC result in drastic amounts of cell death. Notably, VPA induced cell death already at concentrations of 1 mM.

Long-term differentiation

The neuronal phenotype of vehicle (DMSO, 48 hours) or SAHA (0.5 µM, 48 hours) treated cells was further characterized using Map2 immunocytochemisty. SAHA treatment increased the number of Map2 expressing cells compared to controls (DMSO) by 185 % (Fig. 15, Table 5). In addition, prolonged differentiation periods of 28 days allowed identifying neurons with a GABAergic (GAD65/67) or dopaminergic (TH) phenotype. These markers co-localized with βIII tubulin expression (Fig. 15). SAHA treatment increased numbers of TH+/βIII tubulin+ cells from 21.3 % ± 1.2 % in controls to 33.1 % ± 1.4 %. Numbers of GAD65/67+ / βIII tubulin+ cells increased from 17.0 % ± 1.3 % to 29.3 % ± 1.6 % (Table 6, Fig. 16). No cells were found to express vGlut1 (not shown).

Substance	Total # cells	# neurons	# astrocytes	% neurons (± SEM)	% astrocytes (± SEM)
DMSO	2539	420	1027	16.4 ± 0.6	40.7 ± 1.5
SAHA	2082	636	838	30.5 ± 1.1	40.5 ± 1.4

Table 5: Numbers of Map2 positive cells in control and SAHA treated cultures. Neurosphere-derived cells were exposed to vehicle (DMSO) or 0.5 µM SAHA for 48 hours and differentiated for 7 days altogether. Given are numbers and percentages of Map2+ neurons and GFAP+ astrocytes from three independent experiments. SEM, standard error of the mean.

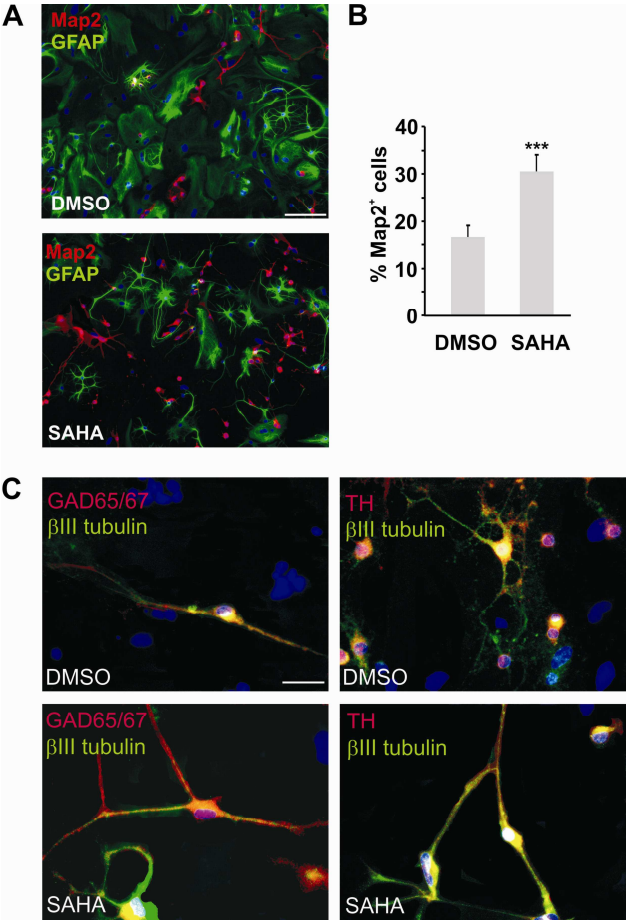


Figure 15: Differentiation into mature neurons after SAHA treatment. A, Immunostaining for Map2 positive neurons (red) and GFAP positive astrocytes (green). Neurosphere-derived cells were treated with vehicle (DMSO) or SAHA (5µM, 48 hours) and differentiated for seven days altogether. Nuclei are counterstained by Hoechst (blue). Scale bar, 20 µm. **B**, Quantitative analysis of the cells differentiated in **A**. Given is the percentage of Map2+ neurons compared to the total cell population (see table 5). Asterisks indicate statistical significance (t-Test, $p<0.01$). **C**, Identification of GABAergic (GAD65/67, red) and dopaminergic (TH, red) neurons in vehicle (DMSO) and SAHA (5 µM, 48 hours) treated neurosphere-derived cells that were differentiated for 28 days altogether. Both markers co-localize with βIII tubulin. GABAergic as well as dopaminergic cells are present in DMSO as well as SAHA treated cells. Scale bar, 10 µm.

Substance	Total # cells	# TuJ+ GAD+	% TuJ+ GAD+ (± SEM)
DMSO	9068	1511	16.96 ± 1.28
SAHA	8203	2358	29.25 ± 1.58
Substance	**Total # cells**	**# TuJ+ TH+**	**% TuJ+ TH+ (± SEM)**
DMSO	14188	2908	21.27 ± 1.24
SAHA	7147	2329	33.07 ± 1.43

Table 6: Cell numbers of neurosphere-derived cells after 28 days of differentiation. SPCs were treated with DMSO or SAHA for 48 hours at the onset of differentiation. TuJ, βIII tubulin. GAD, glutamic acid decarboxlase. TH, tyrosine hydroxylase. SEM, standard error of the mean.

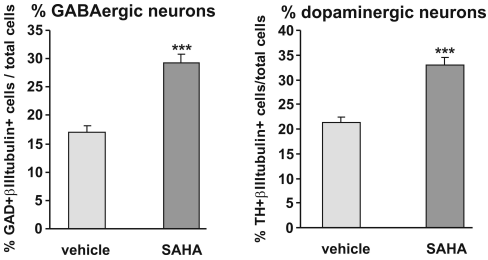


Figure 16: Bar graph of differentiation into mature neurons. SPCs were treated with DMSO (vehicle) or SAHA as stated above (cf. Table 6 and text, p. 51). Asterisks indicate level of significance (t-Test, $p<0.0001$).

Gene expression analysis

Gene expression targeted by HDAC inhibitors was analyzed by quantitative real-time PCR. The activating transcription factor 5 (Atf5) was found significantly downregulated following iHDAC treatment. Atf5 has been implicated in maintenance of stem cell proliferation and Atf5 expression levels decrease with onset of differentiation (169, 170). Previous reports pointed to the transcription factor NeuroD and the cell-cycle control gene Cyclin D2 to be upregulated by HDAC inhibitors (81, 82). These findings were confirmed by quantitative RT-PCR analysis (Fig. 17). Expression of Cyclin D3 was unaffected while that of Cyclin D1 was downregulated. Furthermore, a novel gene induced by HDAC inhibition was identified. SAHA, M344, MS-275 and VPA were able to up-regulate the B-lymphocyte translocation gene 3 (Btg3) in neurosphere-derived cultures. Btg3 is a cell-cycle inhibitory gene and

belongs to the PC3/Btg/TOB family of antiproliferative genes (171). Another member of this family (Btg2) previously identified in neurogenesis (172, 173) was not affected by HDAC inhibitor treatment (Fig. 16). In contrast to increased levels of neuronal differentiation markers, significantly lower mRNA expression of oligodendrocyte lineage transcription factor 2 (Olig2) was found. The downregulation of Olig2 is in accordance with decreased numbers of CNPase immuno-reactive oligodendrocytes (Figs. 13 and 17).

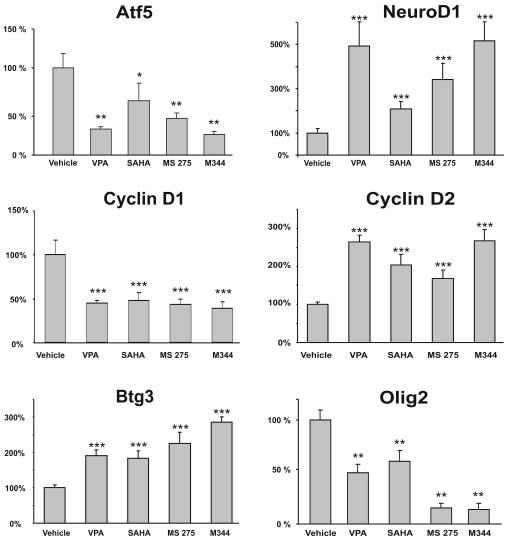


Figure 17: Quantitative real-time RT-PCR for iHDAC induced genes. Following treatment of neurosphere-derived cells with iHDAC in optimized concentrations and exposure times (see text), total RNA was extracted. Quantitative real time RT-PCR for NeuroD1, Cyclin D2 and Btg3 revealed significant induction by HDAC inhibitors, while expression of Atf5, Cyclin D1 and Olig2 was decreased. Given are relative expression levels compared to GAPDH. Asterisks indicate levels of statistical significance (one-way ANOVA, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Culture model for adult human neural precursor cells

Human hippocampal tissue was obtained from surgical specimens from patients with temporal lobe epilepsy resections at the University Hospital Erlangen as well as from autopsy-derived tissue. Hippocampal tissue was laminated and a slice of approx. 2 mm thickness containing the dentate gyrus was freed from blood and white matter, while the rest of the tissue was processed for neuropathological examination. After mincing with a scalpel blade, tissue was dissociated by enzymatic digestion (in PPD solution, cf. Methods p. 32f.). The resulting cell suspension was washed twice in PBS and centrifuged in a 0.9 M sucrose density gradient (174). Human multipotent adult neural progenitors were expanded as monolayer cultures adherent to laminin/poly-ornithine coated plates in N2 medium supplemented with 5 % FCS, EGF, FGF2 and LIF (‘human expansion medium’). Markers of ‘stemness’ such as Nestin and Musashi1 were expressed under these conditions (Fig. 18).

Growth factors were added every third day and medium exchange was performed once per week. Cells were passaged when reaching confluence. Passaging was accompanied by – sometimes severe – cell loss, as was revealed by total cell counts (data not shown). Human cultures and clinical data are summarized in table 7.

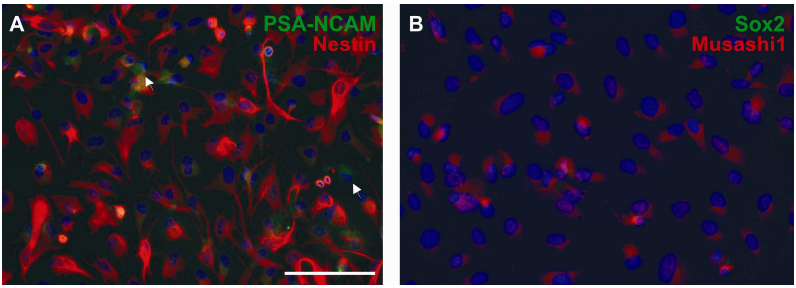


Figure 18: Expression of stem cell markers in cultured human precursor cells. Human hippocampal precursor cells (from culture H070507) were fixed during expansion culture and immunostained for different stem cell antigens. **A**, most cells express the stem cell marker Nestin (red) while only very few cells express PSA-NCAM (arrows). Nuclei are counterstained with Hoechst (blue; also in B). Scale bar, 100 µm (applies also to B). **B**, While Musashi1 (red) expression is abundant; Sox2 (green) is not expressed.

Culture	Gender	Age	Hemisphere	Specimen	Diagnosis
H190606	female	55	right	surgery	TLE, AHS, GCD
H260606	female	37	left	surgery	TLE, AHS, GCD
H201006	female	58	left	autopsy	schizophrenia
H021106	female	99	left	autopsy	cardiac arrest
H301106	female	39	right	surgery	TLE, gliosis
H051206	female	37	right	surgery	TLE, GCD
H050207	female	37	left	surgery	TLE, AHS, GCD
H220207	female	41	right	surgery	TLE;
H070507	female	31	left	surgery	TLE, AHS, GCD

Table 7: Clinical data of human patients included in this study. Notably, all patients of whom tissue was obtained were female. The mean age at time of surgery was 39.4 years. Importantly, some patients suffered from Ammon's horn sclerosis, while others did not. TLE, temporal lobe epilepsy. AHS, Ammon's horn sclerosis. GCD, granule cell dispersion.

Induction of neuronal differentiation in adult human neural precursor cells

For differentiation induction, adult human hippocampal precursors were plated onto laminin/poly-ornithine coated cover slips and grown in human expansion medium (cf. Methods, p. 30f.) for 48 hours. In contrast to rodent precursor cells, spontaneous differentiation of human precursors after growth factor withdrawal is extremely low (approx. 0.1 % neurons and glia, data not shown) with most cells remaining in a Nestin-positive, undifferentiated state (data not shown).

Several protocols were tested for induction of neuronal differentiation. Two recently described protocols for neuronal differentiation of human cells, one using a combination of IBMX, dibutyryl cyclic AMP and NGF (175) and the other BDNF and retinoic acid (176) were compared to a newly developed protocol that involved combined treatment with forskolin, NGF and SAHA (Fig. 19). Differentiation was assessed according to the following criteria: gross changes in cellular morphology were examined by light microscopy. Only when the morphology changed from flat cells to cells supporting processes, further evaluation by fluorescence immunocytochemistry for lineage determination was performed and neuronal cell numbers were counted.

Using combined treatment with BDNF and retinoic acid, no changes in cellular morphology could be observed (data not shown), while both IBMX and forskolin treatment induced complex neuronal morphology within few hours following treatment (Fig. 19 C, E). Withdrawal of IBMX after 24 to 72 hours resulted in reversal of the complex morphology to flat, undifferentiated cells. However, continued exposure over six days to IBMX resulted in significant cell loss (Fig. 19 D).

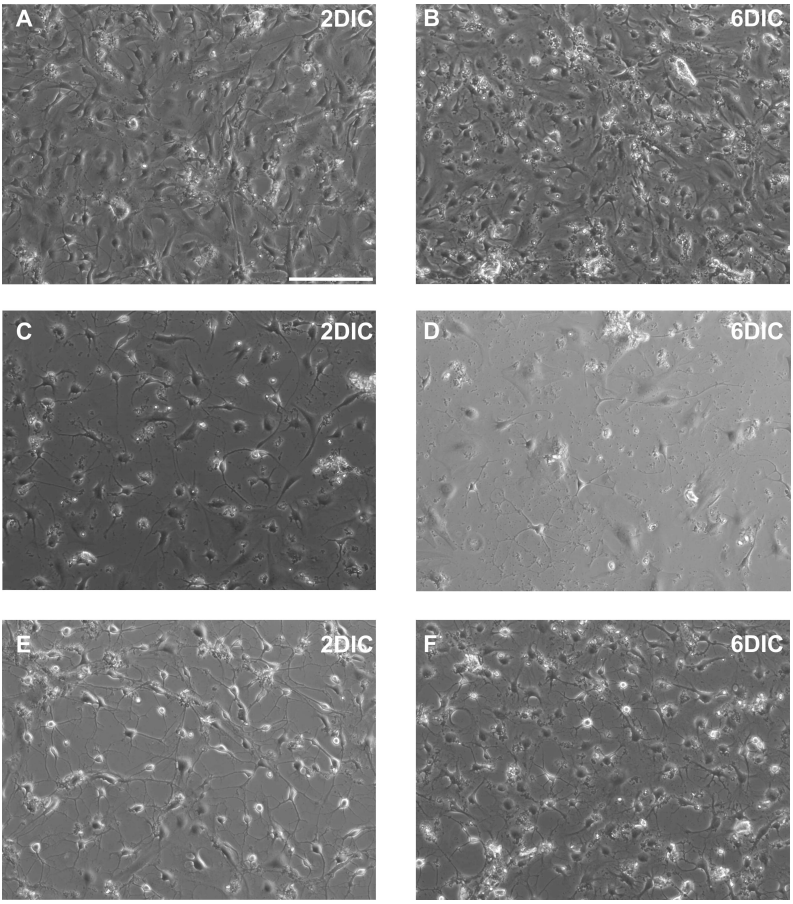


Figure 19: Differentiation of human precursor cell cultures. Human precursor cells (from a representative experiment; culture H070507) were differentiated according to the above-mentioned protocols or by growth factor withdrawal. Replacement of expansion medium with growth factor-free N2 supplemented with 1% FCS (**A** and **B**) resulted in formation of cellular processes. Treatment with IBMX, dibutyryl cAMP and NGF generated cells with neuronal morphology within 2 days (**C**), although only few cells survived treatment for longer periods (6 days, **D**). Combined treatment with SAHA, forskolin and NGF resulted in process-bearing cells of neuronal morphology that survived longer treatment paradigms (**E** and **F**). Scale bar in A, 100 µm.

Morphology changed and expression of neuronal markers (βIII tubulin and Map2) increased significantly when human hippocampal progenitors were treated with a combination of 5 µM forskolin and 1 µM SAHA (up to 70 %, Table 8 and Fig. 20). Neuronal numbers were quantified by manual counting following immunostaining for Map2. Cell numbers were counted in 10 randomly placed visual fields (0.6 mm^2) per experiment (Table 8).

Differentiation into Map2-positive neurons after treatment with SAHA and forskolin was compared between three AHS-derived and four non-AHS derived cultures. Altogether, non-AHS-derived cells were able to differentiate into higher percentages of neurons, while AHS-derived cultures showed ineffective neuronal differentiation (Fig. 20).

Culture	**Differentiation conditions**	**# VF**	**# total cells**	**# $Map2^+$**	**% $Map2^+$ (± SEM)**
H190606	untreated	20	2825	103	3.5 ± 0.8
	SAHA (48h)+ forskolin	30	2193	87	4.6 ± 2.9
H260606	untreated	10	2419	16	0.8 ± 0.3
	SAHA (48h)+ forskolin	30	1541	25	1.7 ± 0.5
H201006	untreated	10	1567	9	0.5 ± 0.2
	SAHA (48h)+ forskolin	30	1903	1333	74.1 ± 2.7
H021106	untreated	10	818	97	14.5 ± 4.6
	SAHA (48h)+ forskolin	10	632	368	56.7 ± 5.6
H301106	untreated	10	1533	40	2.7 ± 0.8
	SAHA (48h)+ forskolin	30	1788	1272	74.1± 2.9
H051206	untreated	10	1689	0	0 ± 0
	SAHA (48h)+ forskolin	10	632	421	63.3 ± 8.6
H050207	untreated	20	5575	67	1.3 ± 0.3
	SAHA (48h)+ forskolin	30	1657	1056	65.6 ± 2.4
H220207	untreated	20	1268	226	20.1 ± 2.5
	SAHA (48h)+ forskolin	30	1619	598	40.0± 3.5

Table 8: Differentiation of human progenitor cells. Precursor cell cultures were differentiated by growth factor withdrawal ('untreated') or by combined treatment with SAHA and forskolin. Numbers of differentiated neuronal cells were quantified after seven days of differentiation. # VF, number of visual fields counted. Ten visual fields were counted per independent experiment.

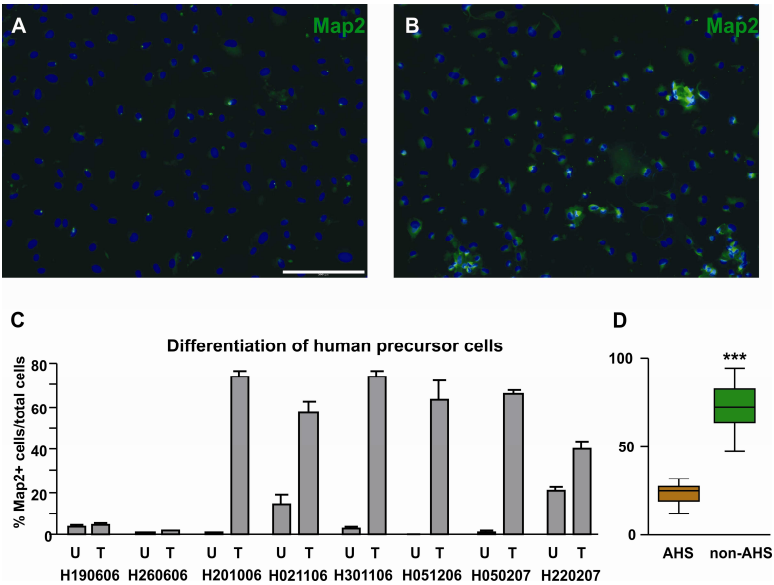


Figure 20: Quantification of neuronal differentiation from human hippocampal precursor cells. Human hippocampal precursors (from culture H201006) were differentiated by treatment with forskolin and SAHA. **A**, Untreated control. Scale bar, 100 µm (applies also to B). **B**, Treatment with forskolin and SAHA induced expression of the neuronal marker Map2 (green) as well as neuronal morphology in these cells. Nuclei are visualized by Hoechst staining. **C**, Quantification of forskolin and SAHA treated cell numbers showed in 6 out of 8 cultures analyzed a dramatic increase of neuronal differentiation. U, untreated control. T, treatment with forskolin and SAHA. **D**, Differentiation into Map2-positive neurons from AHS and non-AHS derived cultures. AHS cultures are H190606, H260606 and H050207. Non-AHS cultures are H201006, H021106, H301106 and H051206. Asterisks indicate statistical significance (t-Test, $p<0.0001$).

Mature neuronal phenotypes

Differentiation into mature neurons was achieved by pre-treating human precursor cells with a combination of the midbrain-fate inducing cytokines Shh and FGF8 (177) and the iHDAC SAHA for 48 hours, followed by treatment with forskolin and NGF for 12 days. Mature neurons were identified after 14 days of differentiation by immunofluorescence double staining for a combination of the markers NF-L, GAD65/67, TH, Map2, βIII tubulin and NeuN (Fig. 21). NeuN-

positive neurons were quantified and the capacity of different treatment paradigms for the induction of NeuN expressing neurons were compared (Table 9, Fig. 22). Highest numbers of mature neurons were found after treatment with Shh, FGF8, SAHA, forskolin and NGF.

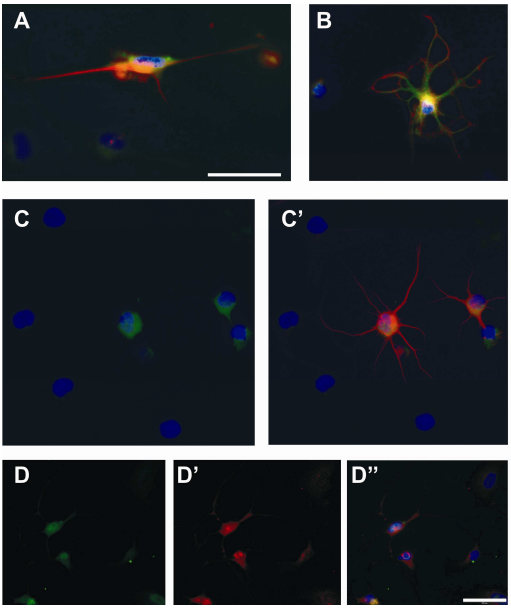


Figure 21: Expression of mature neuronal markers in human precursor cells. Human hippocampal precursor cells were differentiated using the above-mentioned protocol for 14 days *in vitro*. Cells from the culture H051206 expressed neurofilament (red) and NeuN (green) (A) as well as βIII tubulin (green) and tyrosine hydroxylase (red) (B). Scale bar in A (50 µm) applies also to B and C. Differentiated H070507 cells expressed NeuN (green, C and C') and βIII tubulin (red, C') as well as Map2 (green, D and D'') and GAD65/67 (red, D' and D''). Nuclei are stained by Hoechst (blue). Scale bar in D'', 100 µm.

Culture Conditions	# total cells	# NeuN+	% NeuN+ (± SEM)
Forskolin + NGF	2127	65	3.2 ± 0.6
SAHA + forskolin + NGF	1310	48	3.6 ± 0.5
Shh + FGF8 + forskolin + NGF	2598	74	2.9 ± 0.6
Shh + FGF8 + SAHA + forskolin + NGF	1593	100	6.4 ± 0.8

Table 9: Expression of mature neuronal markers in one representative human culture. H070507 cells were treated with a combination of Shh, FGF8, SAHA, forskolin and NGF. NeuN expression was analyzed by immunofluorescence microscopy after 14 days of differentiation. A total of 10 visual fields were examined per culture condition.

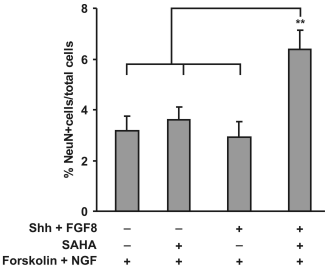


Figure 22: Quantification of NeuN expressing cells. H070507 cells were treated with combinations of Shh, FGF8, SAHA, forskolin and NGF and differentiated for 14 days. Combined treatment with all substances increased the expression of NeuN approx. two-fold over other paradigms. Asterisks indicate statistical significance ($p<0.01$, t-Test). See also table 9.

Generation of cell lines derived from the rodent subventricular zone

Using a neurosphere culture model in which cells were seeded at a density of 20,000 cells per cm^2 into anti-adhesive cell culture plates, adult rodent SVZ precursor cells were expanded with two passages per week. During the first eight passages all spheres showed characteristics of neural precursor cell cultures, i.e. multipotent differentiation (data not shown; see also (178)). Between passages eight and ten, cultured cells changed their morphological appearance and formed atypical cellular aggregates different from genuine neurospheres (Fig. 23). This was accompanied by their ability to expand also under differentiation conditions, i.e. growth factor (EGF, FGF2) withdrawal in the presence of serum. However, the expressions of neuronal (βIII tubulin, Map2) and glial markers (GFAP) were unaffected under differentiation conditions (Fig. 23).

The derived cell line (designated "R2303" henceforth) could be passaged for more than 110 times without a decline in the doubling rate of approx. three days. Repeating the above-specified experimental approach in an independent setting generated another cell line (termed "R2902").

Both cell lines can be expanded indefinitely and express neural stem cell markers (i.e. Sox2, Musashi-1, CD133/prominin-1 and Nestin) under expansion conditions and neuronal and glial markers (i.e. βIII tubulin, Map2, GFAP) under differentiation conditions (Fig. 23). Moreover, RT-PCR revealed expression of neural stem cell associated genes in both cell lines (i.e. Nestin and Musashi-1). However, expression of these stem cell markers continued under differentiation conditions (Fig. 23).

The existence of a “side population” has been confirmed in both adult and cancer stem cells (179, 180). Its higher drug efflux ability is caused by ABCG2 drug transporter expression (180), resulting in low staining capacity for the cell permeable DNA binding dye Hoechst 33342. FACS analysis of R2303 cells detected a significant side population, which could be blocked using Verapamil, an ABCG2 inhibitor (Fig. 23).

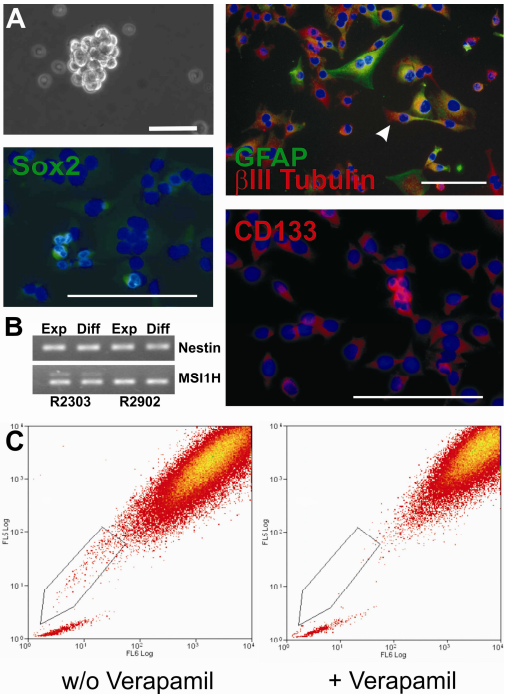


Figure 23: Generation of cell lines. **A**, Expansion of SVZ precursor cells over 10 passages *in vitro* resulted in morphologically homogenous cultures (termed R2303 and R2902) that presented as spheroid aggregates in suspension cultures. Scale bar, 50 µm. Differentiated R2303 and R2902 cells are immunopositive for neuronal (βIII tubulin) and glial (GFAP) markers. While most cells expressed either neuronal or glial markers, some cells were positive for both (arrowhead), indicating a possible transition phenotype (101). Furthermore, R2303 cells express stem cell markers Sox2 and CD133 in culture. Scale bars, 50 µm. **B**, RT-PCR for stem cell associated genes revealed that R2303 and R2902 cells express Nestin and Musashi-1 (MSI1H) mRNA *in vitro* both under expansion (i.e. sphere culture, Exp) and differentiation (i.e. growth factor withdrawal and serum addition, Diff) conditions. **C**, Side population analysis. R2303 cells were stained with Hoechst 33342 and analyzed on a MoFlo Cell Sorter. A significant side population consisting of 0.3 % of all cells was found (100,000 events analyzed). The side population could be suppressed by Verapamil treatment.

Chromosomal analysis

Chromosomal analysis of both cell lines at different passages revealed multiple numeric and structural chromosomal aberrations as well as an overall genomic instability (Fig. 24). Eighty percent (R2303) and fifty-two percent (R2902) of analyzed metaphases had 38 chromosomes compared to 42 for normal rats. While a high-grade chromosomal mosaicism was found, some chromosomal aberrations were present in all mitoses analyzed. These included derivative chromosomes 6, 11 and 14 as well as an isochromosome 2. R2902 cells also showed a trisomy 7, which was not found in R2303 cells. Of note, both cell lines showed one derivative chromosome 14 (inset, Fig. 24).

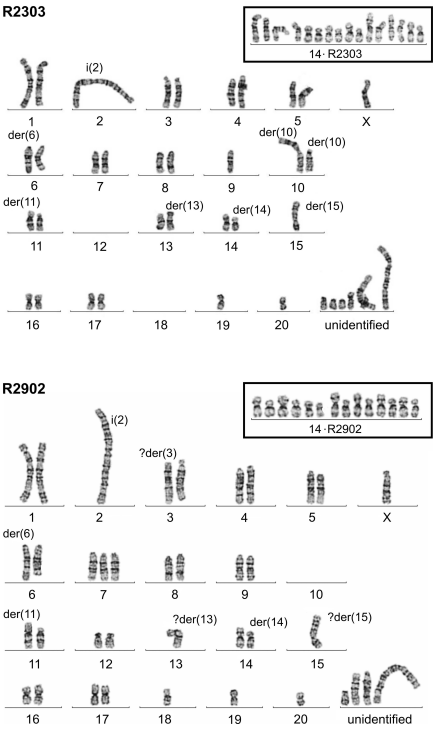


Figure 24: Karyotyping of cell lines. Karyotyping of R2303 and R2903 cells revealed multiple structural and numeric chromosomal aberrations. All metaphases analyzed in both cell lines had an isochromosome 2 and derivative chromosomes 6, 11 and 14. Several meta-phases showed further derivative chromosomes 8, 10, 13 and 15 compatible with a high-grade mosaicism. Some chromosomal derivatives could not be identified. Inset: Pair-wise comparison of chromosome 14 from different metaphases. In each sample, derivation in the short arm of one chromosome was recognized.

Tumorigenicity of subventricular zone cell lines

Transplantation of cell lines into rat brain

R2303 cells were transplanted into the right striatum of syngenic and immunocompetent male adult rats (50,000 cells in a total volume of 5 µl). Extensive tumor masses were recognized in 4 out of 5 transplanted animals. Histopathological examination revealed streak necrosis, invasive growth (Fig. 25) and expression of neuronal (Map2ab, βIII tubulin) as well as undifferentiated (Nestin) markers (Fig. 25). No GFAP immunoreactivity was observed in these cases. These features are compatible with experimentally induced gliomas (181-183).

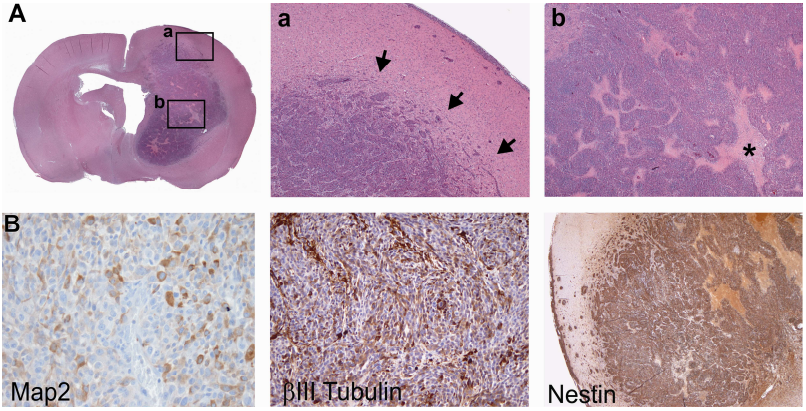


Figure 25: Tumor formation after R2303 transplantation. A, Transplantation of 50,000 R2303 cells into the right striatum of adult male Wistar rats resulted in the formation of solid tumors (27 days post transplantation depicted). **a**, higher magnification (12.5x) of the upper inset reveals invasion into surrounding brain parenchyma (arrows). **b**, higher magnification (40x) of the lower inset reveals streak necrosis (asterisk). **B**, Immunohistochemistry shows the expression of neuronal (Map2, 200x and βIII tubulin, 100x) and un-differentiated markers (Nestin, 12.5x).

Further immunohistochemical analysis identified two distinct populations of tumor cells, one characterized by nuclear accumulation of p21, the other expressing the proliferation marker Ki-67 (Fig. 26). Double fluorescence staining confirmed that both populations are mutually exclusive (Fig. 26). No nuclear accumulation of p53 or Olig2 was detectable (data not shown).

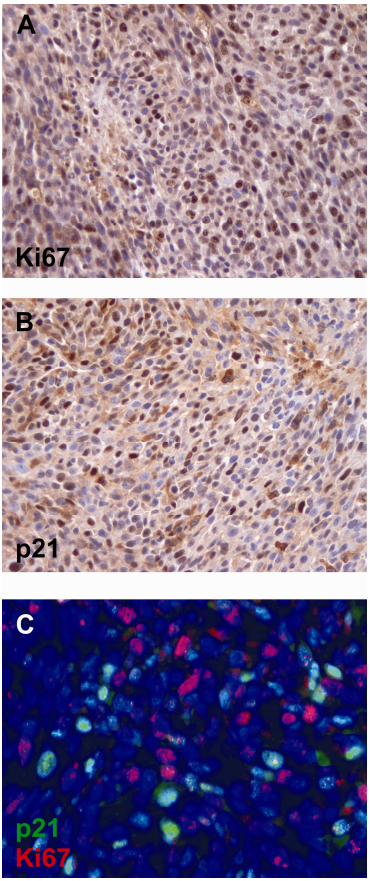


Figure 26: Proliferation and cell cycle arrest within R2303-derived tumors. A, Staining for the proliferation marker Ki67 revealed a high proliferative index of R2303-derived tumors, compatible with high malignancy (200x). **B**, A population with nuclear accumulation of the cell-cycle regulator $p21^{Waf1/cip}$ was revealed by immunohistochemistry. Nuclear p21 accumulation normally results in cell cycle arrest (200x). **C**, Fluorescence immunostaining for p21 (green) and Ki67 (red) showed both populations to be mutually exclusive, pointing to a proliferative and a senescent subpopulation of tumor cells (400x). Blue: nuclear counterstaining (Hoechst).

Gene expression analysis

To further characterize cell lines R2303 and R2902, gene expression of oncogenes and tumor suppressor genes frequently associated with malignant brain tumors was examined, including EGFR, Olig2, p21, p53, PDGFRα and PTEN. The rationale was based on previous chromosomal analysis identifying prominent structural and numeric aberrations (see above). In addition, EGFR and PFGFRα are located on rat chromosome 14, which was found aberrant in all studied metaphases. All target genes listed above were expressed in R2303 and R2902 cell lines (data not shown). However, quantitative real-time PCR identified significantly different expression levels when comparing R2303 and R2902 cell lines with syngenic primary SVZ precursors using both expansion as well as differentiation conditions. There were no differences for EGFR, Olig2, p53 and PTEN. Significant upregulation of p21 was detected for R2303 and R2902 cells under differentiation conditions ($p<0.001$, two-way ANOVA, data not shown). Furthermore, reduced levels of PDGFRα expression were found during differentiation of normal rodent precursor cells, while expression levels remained unchanged in R2303 and R2902 cell lines under expansion or differentiation conditions ($p<0.001$, two-way ANOVA; Fig. 26). Prominent PDGFR staining can also be detected immunohistochemically in R2303 transplanted tumors (Fig. 27). RT-PCR revealed that both cell lines and normal precursor cells express the growth factor subunits PDGF–A and –B *in vitro* (Fig. 27).

siRNA knockdown of PDGFRα

To analyze whether the continued expression of PDGFRα is sufficient for the proliferation of cancer stem cells in culture, a knockdown experiment for the PDGFRα in R2303 cells was performed. 5,000 cells were seeded into 96 well plates and transfected with 5 nM of two different siRNA molecules designed against rat PDGFRα. R2303 cells transfected with oligonucleotides with no sequence similarity to

any known gene were used as controls. Cell growth was quantified using the MTT assay (159) seven days after transfection (n=8 each). Cell viability compared to mock transfected controls was decreased by 50 % and 85 %, respectively, in siRNA-transfected cells ($p<0.01$, t-Test; Fig 27). Reduced PDGFR protein levels were found when immunostaining siRNA-transfected cells 48 and 96 hours after transfection (Fig. 26). Furthermore, inhibition of PDGFR signaling by a specific receptor tyrosine kinase inhibitor (4-(6,7-Dimethoxy-4-quinazolinyl)-N-(4-phenoxyphenyl)-1-piperazinecarboxamide) resulted in R2303 cell growth reduction in low micromolar concentrations (n=8; IC_{50} = 1.4 µM; Fig. 27C) similar to PDGFRα knock-down. RT-PCR revealed that R2303 cells as well as stem/progenitor cells express PDGF–A and –B in culture both under expansion and differentiation conditions (Fig. 27).

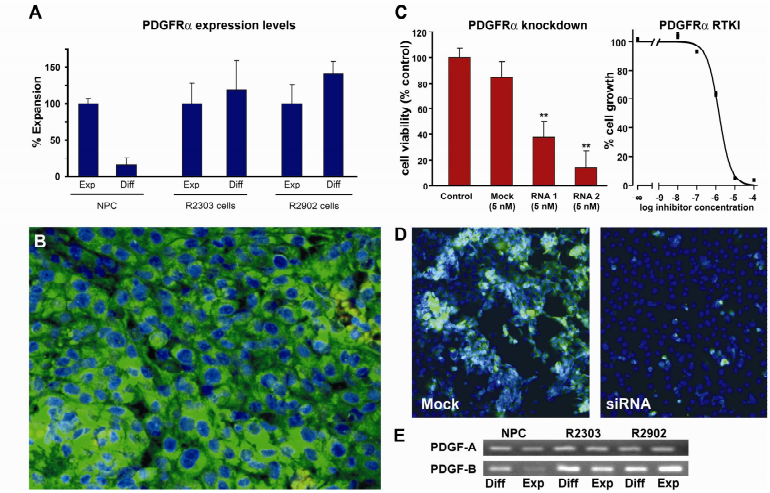


Figure 27: Gene expression analysis of tumor cell lines. A, Quantitative real-time PCR of normal neurospheres as well as R2303 and R2902 cells under expansion (Exp; sphere culture) and differentiation (Diff; growth factor withdrawal and serum addition) conditions revealed differences in PDGFRα expression levels (normalized to expansion conditions). Normal SVZ precursor cells downregulate the PDGFRα upon induction of differentiation ($p<0.001$, two-way ANOVA), while both R2303 and R2902 cells show no significant differences in expression of PDGFRα. Target gene expression was normalized to the housekeeping gene GAPDH. **B**, Fluorescence immunostaining for PDGFR (green) within R2303 induced tumors revealed prominent expression of this receptor (400x). **C**, siRNA knockdown of the PDGFRα *in vitro* as well as treatment with a PDGFRα specific receptor tyrosine kinase inhibitor (RTKI) resulted in growth inhibition of R2303 cells. Data are from a cell viability assay (MTT, n=8). Control: R2303 cells incubated with lipofectamine. Mock: R2303 cells transfected with nonsense oligonucleotides. RNA1 and RNA2: R2303 cells transfected with siRNA directed against different parts of the PDGFRα sequence. Asterisks indicate statistical significance (Student's t-Test, $p<0.01$). A PDGFRα RTKI reduced cell growth dose-dependently. Depicted is a best fit curve for the induction of growth inhibition in R2303 cells. **D**, Immunofluorescence staining for PDGFR (100x). R2303 cells were either mock or PDGFRα-siRNA transfected (using siRNA2 of the experiment in C) and immunostained for PDGFR 96 hours after transfection. **E**, RT-PCR for PDGF–A and –B revealed that both subunits are expressed by R2303 and R2902 cells as well as normal precursor cultures (NPC).

Discussion

The ability of the adult mammalian brain to generate new neurons under physiological as well as pathophysiological conditions has been established not more than fifteen years ago by Reynolds and Weiss (3), although the first reports date back nearly half a century (2). Meanwhile, various brain areas have been identified in which adult quiescent stem cells hibernate throughout life and will be activated upon specific molecular signaling pathways, electrical activity such as seizures or hypoxic damage, i.e. stroke (8, 111, 114). These intriguing findings have fostered the scientific approach towards regenerative treatment modalities in CNS disorders (128, 184). However, specific developmental pathways for the selective differentiation of neuronal and glial progenies are only beginning to be understood (71, 185-188). The epochal discoveries that embryonic stem cells can be used to generate neuronal as well as glial cell populations in large enough amounts have further influenced reconstructive approaches in CNS disorders (189, 190). Ethical considerations about the use of embryonic stem cells obtained from human beings, however, raised the demand for alternative cellular resources. In this respect, adult stem cells may open new avenues for such treatment modalities in regenerative medicine.

Isolation and propagation of adult brain precursor cells

Adult stem cells have been isolated from various brain regions, both from living specimens and from post-mortem tissue (20, 191, 192). These cells can be expanded *in vitro*, providing a practically inexhaustible resource to study pathways of proliferation, differentiation and integration. Such pathways may not only be triggered by developmentally regulated transcription factors and signaling cascades, but also by epigenetic modulation of chromatin structure. Cytosine methylation as well as histone (de-) acetylation have a major impact on gene expression profiles within a particular cell population (83, 91). There is increasing evidence that these mechanisms play a regulatory role during development, adolescence and aging (193-195).

For this study, adult precursor cells were obtained from the SVZ of postnatal rodents. Self-renewal properties were examined testing the capacity of clonally derived (either by neurosphere assay or by single cell sorting) neurospheres to form secondary spheres *in vitro*. Furthermore, self-renewing neurospheres had the potency to generate glial and neuronal progenies after growth factor withdrawal and serum addition. These properties correspond to previously described characteristics of adult stem cells, either obtained from SVZ or dentate gyrus of rodent and primate brains (192, 196, 197). A potential contamination of cell cultures with fibroblasts can be excluded, as normal brain parenchyma is free of fibroblast cells. Furthermore, fibroblasts are not able to grow in suspension culture and the culture media used for the expansion and differentiation of neural precursor cells are non-permissive for the growth of mesenchymal cell lines, such as NIH3T3, PA6 and L cells (F. Siebzehnrübl, unpublished observation).

Enhanced neuronal differentiation by epigenetic modulation

The availability of pharmacological compounds interfering with histone deacetylases offers the exciting opportunity to study these events also in adult stem cell derived progenies. This approach was tested in neurosphere-derived cells using different inhibitors of HDAC, namely MS-275, M344, SAHA and VPA.

All compounds were able to induce growth arrest and morphogenetic differentiation in a malignant glioma cell line in a concentration-dependent manner. The observed IC_{50} values in this paradigm were markedly different from recently published data (198). This may be due to the specific cell culture model. It should be stressed, however, that the IC_{50} values in the present study reflect tumor cell growth inhibition and not a direct inhibition of deacetylase enzymes.

Neurosphere-derived cells incubated with above-mentioned iHDAC revealed the impact of epigenetic modulation on fate determination and neuronal differentiation *in vitro*. All tested iHDAC had the propensity to significantly induce neuronal differentiation (Table 4 and Figs. 13 and 15). However, the capability of VPA to modulate neuronal differentiation in fetal SVZ as well as adult hippocampal stem cells has been previously described (81, 82). Substantially higher numbers of neurogenesis were reported in these studies, which may result from different protocols applied for SPC expansion and cellular differentiation.

Here, highly specific inhibitors of HDACs were applied, i.e. SAHA, MS-275 and M344, which in this study were superior to VPA in inducing neuronal differentiation. This effect was found to be concentration-dependent, requiring only low micromolar amounts of

second-generation iHDACs. This observation is compatible with chromatin modification by histone acetylation as important mechanism for neuronal differentiation. Moreover, inhibition during the first 24 hours of differentiation is sufficient to induce neurogenesis as well as diminish oligodendroglial lineage determination. This indicates an early fate decision in SPC differentiation. Higher concentrations of HDAC inhibitors reduced cell numbers rather than inducing further differentiation.

The iHDAC mediated effect on neuronal differentiation is not a selective effect, i.e. resulting from survival of just one cell population, as was shown by a propidium iodide uptake assay. Neurosphere-derived cells were treated with solvent control or iHDAC and the incorporation of propidium iodide was analyzed after 24, 48 and 72 hours, respectively. Propidium iodide uptake compared to vehicle treated controls was unchanged at low iHDAC concentrations used for induction of differentiation, indicating that effects on differentiating cells are not due to an increased amount of cell death. Increased concentrations of 2 - 5 µM showed prominent induction of cell death, most likely by apoptosis (95, 168, 199). Of note, VPA differed from the second generation iHDAC by inducing cell death already in low concentrations (Fig. 14). Thus, a selective effect of VPA cannot be excluded. Pharmacological actions of VPA include reduced Na^+ channel conductance, increase of synaptic GABA and glutamate release, increase of intracellular IP_3 levels, and activation of the transcription factor AP-1 (200-202). More recently, HDAC inhibiting properties of VPA were described (203, 204). VPA reveals isoenzyme selectivity for class I HDACs, but requires considerably large concentrations in the range of 200 µM to 2 mM (205, 206). However, VPA concentrations achievable in the cerebrospinal fluid are usually below HDAC inhibitory levels and cannot be unequivocally increased without risk of toxic side effects (207). More importantly, VPA concentrations that target HDAC activity have a pronounced effect on precursor cell viability (Fig. 14).

The second generation iHDAC are superior in their HDAC inhibiting effects as they are far more selective than VPA, rendering these compounds promising drugs for possible therapeutic applications (208). While SAHA and M344 are non-selective inhibitors of histone deacetylases, restricted specificity of VPA and MS-275 for class I HDACs, i.e. HDAC1, 2, 3 and 8 (203, 204, 209) points to targeted recruitment of transcriptional complexes regulating cell lineage determination. Inhibition of class I enzymes seems sufficient in inducing neuronal differentiation from SPCs. Interestingly, restriction of pro-neuronal genes by the NRSF/mSin3/HDAC1 complex has been reported (96). A likely molecular pathway for neurogenesis includes decay of the NRSF/mSin3/HDAC1 complex, thus promoting neuronal differentiation from neural SPCs. Lineage determination of oligodendrocytes is also dependent on HDAC activity (80). This observation was confirmed in this study using second generation iHDAC. Oligodendrocyte numbers dropped to about 50 % of controls after treatment with iHDAC.

Astrocyte populations were not affected in this experimental approach, although previous studies showed a significant reduction of the astrocytic population after treatment with VPA (81, 82). Several possibilities should be considered. Inconsistencies obtained from different culture models (regarding both origin of used cells, age and/or strain of used animals) may result in variable numbers of endogenous astrocytic progenitors. In addition, cells co-expressing βIII tubulin and GFAP were excluded from quantification, which may contribute to different behavior of astrocytic cell populations reported in other studies (81). Overall, a large variance in astrocyte levels was observed, although astrocyte numbers diverged most at iHDAC concentrations below the most effective concentration, while compound concentrations achieving highest levels of neuronal differentiation produced little or no changes in astrocytic differentiation.

When comparing the numbers of differentiated cells (glial and neuronal) in iHDAC treated and control experiments, a portion of cells do not stain for neuronal, oligodendroglial or astroglial markers. These cells most likely are undifferentiated cells, as some of the differentiated cells are Nestin positive (data not shown). Numbers of undifferentiated cells (identified by neither expression of βIII tubulin, GFAP nor CNPase) fall within the same range for iHDAC and vehicle treated experiments, with the notable exception of SAHA (Fig. 13). Thus, it is conceivable that SAHA has different effects than the other iHDACs tested or has different actions on distinct cell populations. However, as cell numbers were calculated from independent experiments, it is possible that effective numbers of undifferentiated cells deviate from the calculated numbers.

The ability of iHDAC treated cells for long-term differentiation (28 days) was tested (Fig. 12). Both controls and SAHA treated cells showed features of GABAergic (GAD65/76) and dopaminergic (TH) differentiation, while none of the cells expressed markers for glutamatergic neurons (vGlut1, data not shown), thus indicating that iHDAC cannot induce a posterior fate from forebrain precursor cells (cf. Introduction, p. 5ff.). Interestingly, a regulation of the TH promoter by NRSF has been described recently (210). NRSF is able to form a complex with HDAC1 (see above and (96)), which may be disrupted by iHDAC, thus promoting dopaminergic differentiation. Total numbers as well as percentages of control versus SAHA-treated TH+/βIII tubulin+ cells support this hypothesis (Fig. 15 and Table 6). Exposure of SPCs to SAHA also resulted in increased differentiation into GABAergic cells after 28 days (Fig. 15 and Table 6). Thus, short intervals of iHDAC exposure (24 to 48 hours) are sufficient to promote differentiation into mature neurons. Notably, total cell numbers of SAHA treated cells were reduced after long term differentiation over 28 days. As SAHA treatment for 48 hours has no significant effect on cell viability compared to controls, reduced cell numbers most likely reflect induction

of terminal differentiation, while untreated controls continue proliferation for some time after induction of differentiation (211). However, proliferation rates of treated or untreated SPCs have not been analyzed under differentiation conditions in the present study.

It was further analyzed which candidate genes are associated with differentiation inducing effects of iHDAC and, therefore, mRNA expression levels of Atf 5, Cyclin D1, D2 and D3, NeuroD1, Btg2, Btg3 and Olig2 genes were quantified (Fig. 17). A downregulation of Atf5, which is a prerequisite for induction of differentiation in neural stem/progenitor cells (169, 170) was observed. Upregulation of NeuroD1 and Cyclin D2 was implicated in inducing neuronal differentiation using VPA (81, 82). Importantly, these findings were confirmed in this study. Of the other D-type cyclins, Cyclin D3 expression remained unchanged while Cyclin D1 was significantly downregulated. Different functions for the three D-type cyclins have been described and cytoplasmic sequestration of Cyclin D1 is known to enhance neuronal survival (212, 213). Genetic ablation of Cyclin D1 does not influence neurogenesis during development while a Cyclin D2 knockout results in complete loss of proliferating progenitor cells (214). It is conceivable from these studies that (i) Cyclin D2 promotes proliferation of early neuronal progenitors and that (ii) downregulation or nuclear depletion of Cyclin D1 protects newborn neurons from apoptosis. Moreover, Btg3 was identified as promising new target gene for neuronal differentiation in neural precursor cells. Btg3 was prominently upregulated in neural precursor cells after iHDAC treatment. Btg3 is a cell-cycle inhibitor and belongs to the PC3/Btg/TOB family of antiproliferative genes (171). Interestingly, Btg3 is expressed in neurogenic regions of the developing CNS (215) and other members of this protein family (i.e. Btg2) are capable to induce neuronal differentiation (172). However, Btg2 is not regulated in this experimental assay but has the ability to downregulate Cyclin D1 expression (216). Whether this also applies to Btg3 remains to be shown.

Distinct downregulation of Olig2 was found. Olig2 is a bHLH transcription factor expressed in those SVZ precursor cells differentiating into the oligodendrocyte lineage (217). Overexpression of Olig2 in adult SVZ cells leads to a distinct increase of oligodendroglial cell numbers *in vivo* (218). Here, reduction of oligodendrocyte differentiation most likely results from a downregulation of Olig2 by iHDAC (Figs. 13 and 17). Furthermore, dependency of oligodendrogenesis on the HDAC1 enzyme activity has been recently confirmed in zebrafish (219).

In conclusion, inhibition of HDAC complexes in SVZ precursor cells induced a shift in fate decision from oligodendrocytes to neurons, rather than an induction of neuronal differentiation from undifferentiated cells. *In vivo*, transit-amplifying type C cells of the SVZ (29) either differentiate into neuronal cells (upon expression of pro-neuronal genes, such as NeuroD) or into oligodendrocytes (when expressing oligodendrocyte inducing genes, such as Olig2). Decision of which genes are expressed largely depends on transcriptional complexes and recruitment of HDAC enzymes, with HDAC1 being the most important protein (96, 219). Hence, selective iHDAC may provide useful tools to experimentally induce neuronal fate decision in SVZ precursor cells. A single knock-down of the HDAC1 enzyme, however, has no effect on the differentiation of neural precursor cells (O. Hermanson, Stockholm, personal communication) – most likely the other class I HDACs are able to compensate the function of HDAC1.

Protocol for the expansion of adult human hippocampal precursor cells

Most reports about successful isolation and culture of stem cells from human brain have been performed using fetal tissue (220-226). The expansion of adult human neural stem/progenitor cells still remains a challenging issue and only very few studies exist utilizing adult tissue either from surgical resections or from post-mortem brains (175, 176, 227). A number of factors complicate successful studies using adult human neural stem cells: first of all, in contrast to laboratory animals, the human population has a much broader genetic background and thus, differences between individuals will produce comparably greater differences in results. Genetic background accounts for the most prominent disparities in neurogenesis of various adult mice strains (228, 229). Furthermore, human samples normally are obtained either from patients suffering from epilepsy, which has major influence on stem cell proliferation and – possibly – survival (114, 230), or cells are isolated from post-mortem tissue in which case ante-mortem agony and the post-mortem interval may result in stem cell hypoxia and degeneration. Finally, human cells may need a different set of growth factors and cytokines for survival and growth *in vitro* than rodent cells and a systematic analysis of additives in human neural stem cell cultures is still lacking.

For this study, human tissue was obtained both from epilepsy surgery specimens and from autopsy brains. For a complete list of patient data, please refer to table 7. Isolation of precursor cells was performed according to the protocol established for rodent tissue, with the notable exception that cells were purified using a sucrose density gradient (174). Human hippocampal precursors were cultured on laminin/poly-ornithine coated surfaces in the presence of 5 % serum, EGF, FGF2 and LIF. In these conditions, a robust increase of cell

numbers was observed and the cultures could be passaged at least ten times. On the other hand, cellular growth rate was rather slow and passaging was accompanied by significant cell loss, so that cell culture passages often required several weeks. Cell loss may reflect the fact that human brain stem cells are capable of differentiating under expansion conditions (5) and thus, a number of cells underwent senescence.

To characterize undifferentiated human cells, markers of stem and progenitor cells were used. Human precursors were negative for the astrocyte and stem cell marker GFAP (231), but expressed Musashi1 and Nestin. The cells were also negative for PSA-NCAM, which is expressed in fate-committed, migratory neuroblasts (29). While expansion of neuroblasts cannot be completely excluded, it is most likely that human cultures consist of neural precursor cells that are at a differentiation stage between astrocytic stem cell and neuroblast – probably transit-amplifying cells (cf. Fig. 3) (23). Attempts to analyze multipotency by withdrawing growth factors from the cultures were unsuccessful, as levels of spontaneous differentiation in human cells were extremely low (approx. 1 per 1,000 cells; data not shown). It seems that adult human stem cells need further induction to begin differentiation (P. Boukamp, DKFZ Heidelberg, personal communication).

Induction of neuronal differentiation in adult human neural precursor cell cultures

To induce differentiation in human precursor cells, two previously published protocols (175, 176) were compared with a newly developed protocol that was derived from two studies on rodent stem and progenitor cells (81, 178). Common to all three protocols is an induced increase in cyclic AMP levels as a means of inducing differentiation. This was achieved by synthetic analogues in the established protocols and by the adenylyl cyclase activator forskolin in the new protocol. Using a combination of retinoic acid, dibutyryl cAMP and BDNF yielded no morphological differentiation (the first criterion for assessment of differentiated human neural precursor cells) in contrast to recently published data (176), while combined treatment with IBMX, dibutyryl cAMP and NGF as well as treatment with forskolin, SAHA and NGF resulted in neuron-like morphology (Fig. 19) and expression of neuronal markers (Fig. 20, not shown for IBMX). However, exposure to IBMX for more than 72 hours resulted in decreased cell numbers, while continued treatment with forskolin and NGF yielded mature neurons, as assessed by immunofluorescence microscopy (Fig. 21). Importantly, a massive induction of neuronal differentiation was observed when treating differentiating human precursor cells with forskolin and SAHA. While untreated controls did not differentiate (with the exception of H220207 cells which were derived from a ganglioglioma), treatment according to the new protocol yielded up to 70 % Map2 positive neurons (Table 8 and Fig. 20).

Immunostaining of forskolin, SAHA and NGF treated cells for the mature neuronal marker NeuN detected 3.6 % mature neurons, and could be even increased up to 6.4 % by pre-treating differentiating cells with Shh and FGF8. Interestingly, pre-treatment with Shh and FGF8

followed by treatment with forskolin and NGF (without SAHA) did not increase the numbers of NeuN-positive neurons (Table 9, Fig. 22).

Taken together, two of the three protocols tested in adult human hippocampal precursor cells resulted in neuronal morphology, while treatment with retinoic acid, dibutyryl cAMP and BDNF was found ineffective. Treatment with forskolin, SAHA and NGF is superior to IBMX, dibutyryl cAMP and NGF, as continued exposure to IMBX and dibutyryl cAMP resulted in severe cell loss and short-term exposition reverted back to undifferentiated cells (data not shown). Pre-treating differentiating human cells with Shh, FGF8 and SAHA followed by exposure to forskolin and NGF produced mature neurons, as was revealed by immunostaining for NeuN, GAD65/67, neurofilament and Map2 (Figs. 21, 22 and Table 9). To finally establish maturation of differentiated neurons, electrophysiology is required (in cooperation with Dr. B. Berninger, LMU Munich).

When comparing differentiation capacities of adult human hippocampal NPCs derived from AHS patients versus non-AHS patients, it is interesting to note that two out of three cultures derived from AHS patients completely failed to differentiate into neuronal progenies. Statistical analysis of three AHS-derived cultures (H190606, H260606 and H050207) versus four non-AHS derived cultures (H201006, H021106, H301106 and H051206; H220207 was not included as this patient suffered from a ganglioglioma) demonstrated that differences in neuronal differentiation are highly significant ($p<0.0001$, T-test). As one AHS-derived culture differentiated into neurons in similar numbers as non-AHS derived cultures, however, more cultures are needed to determine whether AHS influences differentiation capacities of human hippocampal precursor cells, which might be of major importance for disease pathophysiology considering that AHS and non-AHS epilepsies are separate diseases (232).

To establish true stemness of isolated precursor cells, multipotency needs to be shown. Human precursor cells propagated in this study could be induced to differentiate into neuronal phenotypes. Only very few cells expressed the astroglial marker GFAP after differentiation *in vitro* (<1%, data not shown), regardless of the protocol used for differentiation. Higher numbers of cells were positive for S100β (not shown) and no cells expressed the oligodendrocyte marker CNPase. Several points have to be considered: first, not all astrocytes express GFAP *in vivo* (233). Second, S100β is a marker of mature astrocytes that are not multipotent (166). Third, exposure of *in vitro* cultured human astrocytes to bovine serum may downregulate GFAP expression (U. Bogdahn, Regensburg, personal communication).

It is highly probable that induction of glial differentiation requires a combination of cytokines other than those used for neuronal fate determination. Treatment with BMP2 and LIF (234), however, did not result in expression of GFAP or CNPase (data not shown). As fetal bovine serum is able to deplete GFAP-expression in human glial cell lines (R. Sanchez and M. Götz, LMU Munich, personal communication), the use of human serum may be a good candidate for induction of glial fates.

In conclusion, human hippocampal precursor cells most likely represent transit-amplifying cells that can be induced to a neuronal fate using a combination of cAMP-increase and HDAC inhibition. Still, the protocol for differentiation of adult human NPCs into neuronal cell types may be useful for future clinical trials of stem cell-based therapies and a successful therapeutic approach towards reconstructive cell replacement may also emerge from non-stem cell progenitors.

Generation of rodent SVZ cell lines

For a successful approach to reconstructive therapy strategies using adult stem cells, generating sufficient amounts of transplantable cells is essential. A typical scenario would include isolation of stem cells followed by several rounds of expansion in culture before transplanting stem cells or pre-differentiated progenitors into patients. However, every *in vitro* step increases the chance of genetic and/or neoplastic transformation.

Adult neural precursor cells were expanded over several passages *in vitro* in a culture paradigm that allowed passaging every three to five days, thus reflecting an accelerated propagation. Cellular morphology changed in passage eight and from passage ten on, a homogenous cell line (R2303) emerged. Repeating this paradigm in an independent setting generated another cell line (R2902).

Both cell lines could be expanded as sphere-cultures and differentiated *in vitro* into cells expressing neuronal and/or glial markers. R2303 and R2902 cells expressed stem cell markers under expansion conditions (sphere culture, N2 medium supplemented with growth factors), such as Nestin, Musashi-1, Sox2 and CD133. Differentiation conditions (attached culture on a laminin/poly-ornithine coated surface, N2 medium containing serum without growth factors) failed to induce growth arrest and terminal differentiation in these cells, although differentiation markers (such as GFAP, Map2 and βIII tubulin) were expressed. Karyotyping revealed massive chromosomal aberrations in both cell lines.

Tumorigenicity of adult SVZ precursor cell lines

The origin of brain tumors remains enigmatic, although current hypotheses favor stem cells as source for these malignancies (139-141, 144, 235). Here, two *bona fide* cancer stem cell lines were generated from adult neural precursors following expansion in a culture assay adopted for SVZ stem and progenitor cells. Irrespective of the artificial culture environment, these findings indicate the risk and potential of SVZ precursors to transform into malignant brain tumor-initiating cells.

In a low density culture assay, adult stem cells do not transform after prolonged expansion *in vitro* (149). Culture paradigms employed in this study, however, were designed to achieve rapid expansion of stem and progenitor cells, allowing passaging every three to five days. This will be mandatory if *ex vivo* cell expansion is required within a reasonable therapeutic time schedule, i.e. stem cell transplantation in neurological disorders. It is likely, therefore, that this culture assay particularly recruits transit-amplifying cells, i.e. $GFAP^{-}PDGFR\alpha^{-}$ (see Fig. 28, type C), which are the fastest proliferating cells in the SVZ (236). This cell population may be most sensitive to genomic instability. Interestingly, it has been reported that type C cells of the SVZ can be reverted into multipotent cells in culture and become invasive as a result of prolonged EGF exposure (237). Moreover, no spontaneous transformation of rodent olfactory bulb derived precursor cells was found in this culture model despite several attempts to passage these cells beyond passage eight (unpublished observation), indicating that only cells with self-renewing capacities are sensitive to genomic instability in this culture model.

Indeed, rodent cells are more likely to transform spontaneously in culture than human cells. Nonetheless, precursor cells in this assay transformed already after 10 passages *in vitro* in two independent

settings. This does not exclude the possibility of human precursor cell transformation, which may need more genetic alterations (238). Adult neural stem cells have been shown to be resistant to malignant transformation (149) and olfactory bulb progenitor cells cultured in the same conditions that generated the SVZ-derived cell lines (R2303 and R2902) did not result in spontaneous transformation (data not shown). Thus, SVZ progenitor cells (type C) seem most sensitive to malignant transformation.

Neoplastic transformation during cell culture periods may result from accidental exposure to chemical drugs and/or viruses. Supplement of any other chemical substrate to cell culture media than listed above can be excluded, although the plethora of viruses with transforming potential makes a negative proof almost impossible to obtain. However, adult rodent brain cells were successfully cultured and differentiated during a long period of time (155, 178) without any indication for aberrant behavior of other cells. In addition, expansion of rodent olfactory bulb derived precursor cells did not exceed eight passages and no spontaneous malignant transformation was observed (data not shown).

Detailed histopathological analysis of transplanted tumors detected distinct cell populations, i.e. those with nuclear accumulation of p21. Double immunofluorescence for p21 and Ki67 separated this population from a Ki-67 immunoreactive proliferating cell pool (Fig. 26). Others described prominent expression of Olig2 as inducer of proliferation in gliomas as well as in adult stem cells (239), which could not be confirmed in R2303 and R2902 cell lines. p21 expression has been associated with both cell cycle arrest/senescence and stem cell quiescence (240). The senescence-associated marker β-Galactosidase (156) could not be detected in R2303 cells *in vitro* nor after transplantation *in vivo* (data not shown). Therefore, the issue of p21

accumulating senescent versus quiescent tumor cells will need further clarification.

Proof of stemness in cancer cells remains challenging and several reports postulate CD133 (prominin-1) expression (136) or the detection of a side-population as reliable experimental paradigm (179, 180), but a controversial report has been published (241). Here, CD133 expression was identified in R2303 cells as well as a significant side-population. In addition, both R2303 and R2902 cells expressed the stem cell markers Sox2, Musashi1 and Nestin (Fig. 23). From these experiments it is concluded that both cell lines retain stem cell properties even after prolonged passages *in vitro* (>100).

Several mechanisms of malignant transformation have been recognized in brain tumors and the molecular alteration is likely to associate with specific tumor entities. In this respect, constitutive activation of the PDGFRα pathway is common in several brain malignancies, such as malignant gliomas and primitive neuroectodermal tumors (242, 243). Gene expression analysis in R2303 cells revealed continued expression of PDGFRα after onset of differentiation (Fig. 27). Notably, PDGF is not a component of either expansion or differentiation media in this culture assay. PDGFR expression was confirmed by immunofluorescence microscopy in tumors derived from R2303 grafts. Of note, PDGFRα is located on chromosome 14p, which was found structurally aberrant in one chromosome 14 in R2303 and R2902 cells. Thus it is most likely that lasting expression of PDGFRα in these cell lines results in growth advantage. While PDGFRα is downregulated upon differentiation in normal SVZ precursor cells, its maintained expression in R2303 and R2902 cell lines may account for their continued proliferation and tumorigenicity (Fig. 27). This is substantiated by knockdown of PDGFRα, which results in a reduction of protein levels accompanied by reduced cell growth in R2303 cells. Inhibition of PDGFR signaling by a specific receptor tyrosine kinase

inhibitor (Fig. 27) further supports this notion. This finding is rather intriguing when considering the massive numerical and structural chromosome changes in these cell lines.

Importantly, PDGFRα is expressed on SVZ stem cells (Fig. 28, type B) and cerebroventricular infusion of PDGF as well as overexpression of PDGF in astrocytes and neural progenitors result in malignant glioma formation (147, 148). Furthermore, PDGF–A and –B are expressed by normal as well as transformed stem and progenitor cells *in vitro* (Fig. 27). Therefore, downregulation of PDGFRα may be critical to induce growth arrest upon withdrawal of EGF and FGF2 in culture. Continued expression of PDGFRα may result in growth advantage and lead to cellular transformation.

Although it may seem surprising that the PDGFR pathway has such prominent importance for cell growth considering the large-scale chromosomal aberrations in these cell lines, some PDGF isoforms have been implicated in transformation events (147, 244) and thus, downregulation of the PDGFR during neurogenesis may be a mechanism to protect fast-proliferating transit-amplifying type C cells from malignant transformation. As quiescent stem cells in the subventricular zone express PDGFR, and PDGF expression is widespread throughout the CNS (245, 246), availability of PDGF in this niche is very likely.

A recent report described CD133/prominin-1 expression in adult brain and cancer stem cells (61). While type B cells of the adult SVZ lack CD133 immunoreactivity, both ependymal and glioblastoma stem cells express this marker. These data offer different perspectives. Tumor-initiating cells may either originate from immature, CD133-retaining cells in the mature brain, or from ependymal $CD133^+$ cells. A third hypothesis would imply gain of CD133 expression after malignant transformation. The first concept is, however, unlikely considering the long time-interval that immature cells would have to last between early

postnatal stages, when these cells are formed, and the advanced age, when most brain tumors arise. It cannot be excluded that ependymal cells underwent malignant transformation in this *in vitro* study. However, these cells produce only small, astrogliogenic neurospheres (247), while the sphere cultures presented in this work remain multipotent (178). The isolation of $CD133^-$ cancer stem cells in glioblastomas (248) further argues against $CD133^+$ cells as tumor founder population. With respect to the latter study (248), the impact of CD133 expression remains controversial and may not be an imperative event during malignant transformation. Interestingly, CD133 is also located on the rat chromosome 14, albeit on the long arm that is not structurally aberrant in R2303/R2902 cell lines.

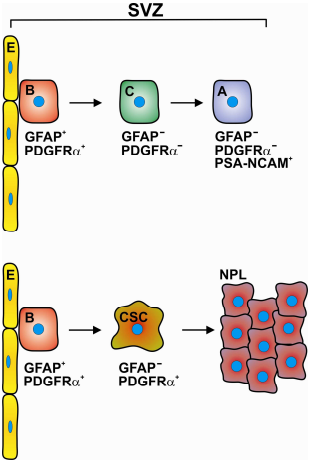


Figure 28: Proposed model for brain tumor progression. In the adult brain, stem cells (type B) of the SVZ give rise to transit-amplifying progenitors (type C), which cease to express GFAP and PDGFRα and further differentiate into PSA-NCAM expressing neuronal progenitors (type A). The GFAP negative cancer stem cell lines R2303 and R2902 continue expression of PDGFRα and likely evolved from type C cells. CSC: cancer stem cell, E: ependyma, NPL: neoplasia. Modified from (236).

These findings are compatible with a tumor progression model in the CNS, which is adopted from the current concept of adult brain stem cell development (14). Type B stem cells reside life-long within their SVZ niche (Fig. 28). These cells are characterized by GFAP and PDGFRα expression (148) and progress into transit-amplifying type C cells, downregulating both GFAP and PDGFRα. Here, transformed type

C cells are proposed as cancer stem cells immortalized in cell lines R2303 and R2902. Lack of GFAP and constitutive PDGFRα expression support this notion and the latter is likely to result from genetic aberrations. Hence, the risk of graft carcinogenesis, which is well documented for embryonic stem cells (131), also applies to therapeutic adult stem cell transplantation strategies. In light of the report of Foroni et al. (149) and the present data, particular efforts have to be dedicated to expansion of adult precursor cells *in vitro*, as paradigms not as restrictive as the neurosphere assay may lead to favored growth of transformed cells. Careful examination for aberrant growth patterns is – therefore – mandatory in any (pre-)clinical reconstructive therapy regimen.

Concluding remarks

Stem cells of the adult brain offer an intriguing perspective for therapeutic replacement strategies in neurological diseases. Although several issues will have to be addressed before therapeutic studies can be initiated, promising results have already been described. Rodent as well as human neural precursor cells can be differentiated into mature neurons and these cells can integrate into existing functional circuitries (175, 249, 250), although for obvious reasons a transplantation into human brain has not been performed yet. In both cases, epigenetic modulation by histone deacetylase inhibition is greatly facilitating neuronal differentiation. Human neural precursor cells can be isolated from surgical specimens as well as from post-mortem tissue. Thus, it is conceivable that by enhancing the organ donation guidelines, enough material for serious therapeutic approaches would be accessible. A small caveat remains, however. Continuous expansion of precursor cells in culture harbors the risk of spontaneous transformation, which in itself is nothing new. Yet, adult stem cells have been thought to be resistant to transforming events. As precursor cell cultures always consist of mixed stem and progenitor cell populations, transformation of a subpopulation is enough to result in tumor formation in graft recipients. This underlines the need for careful examination of stem cell expansion for therapeutic applications.

Outlook

The identification of adult stem cells in the mammalian brain offers a bright perspective for the treatment of hitherto incurable neurologic diseases, although many obstacles still have to be addressed. Candidate disorders for a stem-cell based therapy are diseases in which a defined cell population is affected, such as Parkinson's disease, Multiple Sclerosis or focal ischemia (stroke) (128). Furthermore, therapy of mesial temporal lobe epilepsies may profit from findings of adult neural stem cell research, as these diseases are closely linked to proliferation and differentiation of hippocampal precursor cells (114). Novel therapeutic approaches, such as transplantation of genetically modified stem cells secreting certain growth or survival promoting factors, further expand the spectrum of stem cell medicine towards other diseases (127). Notwithstanding, several questions remain to be addressed before stem-cell based therapies may be tested in pre-clinical trials. These include whether adult stem cells can integrate into higher mammalian brains and differentiate into required cell types, whether transplanted stem cell progenies will benefit graft recipients and whether adult stem cells can induce tumors upon transplantation just as their embryonic counterparts. The ability of precursor cells to transform in culture exemplifies the risk of post-transplantation tumorigenesis. This can be easily overcome by analyzing the tumorigenic potential of cultured precursors, either by a colony-forming assay or by transplantation into nonobese diabetic/severe combined immunodeficient mice. However, cancer stem cells obtained from normal stem and progenitor cells may also offer the possibility to study the events underlying brain tumorigenesis as well as pharmacologic compounds targeting cancer stem cells.

Summary

The discovery of adult stem cells in the mammalian brain has fostered the development of neuro-reconstructive therapy strategies. First reports showed the beneficial potential of adult stem cells grafted into the CNS with neurodegenerative pathology. Before these impressive results can be translated into pre-clinical trials, however, several issues have to be addressed. It is known from studies using embryonic stem cells that the best option for reconstructive cell replacement is the transplantation of pre-differentiated neural progenitor cells, which integrate into the recipient brain and mature along their predetermined lineage. Efficient protocols for the differentiation of adult stem cells are far from being systematically evaluated. Furthermore, only few groups have researched the capacities of adult human neural stem cells for differentiation and transplantation. Finally, it is known from studies employing embryonic stem cells that these cells are tumorigenic when grafted in an undifferentiated state, even if only a few undifferentiated cells contaminate the graft. Adult stem cells are thought to be more resistant to transformation; however, also adult stem cells have to be expanded in culture to attain enough material for transplantation. The potential of adult neural stem/progenitor cells for malignant transformation has not been analyzed so far.

In the present study, a protocol for neuronal differentiation of rodent adult brain precursor cells was established. The use of second generation histone deacetylase inhibitors facilitated the five-fold increase of numbers of differentiated neurons. The differentiating neurons formed GABAergic as well as dopaminergic cells and the yield of both populations could be significantly increased. In contrast, oligodendrocyte numbers were reduced by half while the astrocyte population was not changed.

The molecular basis of induced differentiation was found to be increased expression of pro-neuronal genes, such as NeuroD1 and Cyclin D2. Expression levels of oligodendroglial transcription factors, such as Olig2 decreased. Thus, histone deacetylase inhibitors seem to shift the differentiation from the oligodendroglial to the neuronal lineage. Indeed, a progenitor cell able to differentiate into both subpopulations exists *in vivo*.

The protocol for the induction of neuronal differentiation was subsequently transferred to human hippocampal precursor cells. Human hippocampi were obtained from epilepsy surgery as well as from autopsy tissue. During expansion, human precursor cells expressed markers of stem/progenitor cells, such as Nestin and Musashi-1, while being negative for markers of migratory neuroblasts. Hence, these cells are neural progenitors, so-called “transit-amplifiers”. Three protocols were tested for differentiation of human cells, two of which have been recently published. One of the published protocols and the newly established protocol induced neuronal morphology, whereas cells treated according to the published protocol were lost upon longer incubation and reverted into undifferentiated cells in shorter incubation intervals. In contrast, the new protocol resulted in quantifiable amounts of differentiated cells expressing various markers of immature and mature neurons. Up to 70 % of the induced cells were positive for the neuronal marker Map2, while up to 6.4 % could be stained for NeuN, a protein that is formed exclusively by mature neurons. Contrasting the differentiation capability of cell cultures obtained from patients suffering from Ammon’s horn sclerosis to cultures obtained from non-AHS patients showed a significantly decreased ability for neuronal differentiation in AHS-derived cultures. As the total numbers of cell cultures are relatively low, a general correlation between the differentiation capability of hippocampal precursor cells and Ammon’s horn sclerosis cannot be deduced yet.

Finally, the questions whether adult precursor cells can transform *in vitro* and whether a transformation has consequences for transplantation were addressed. Adult rodent precursor cell cultures changed their phenotype in less than ten passages *in vitro* which was accompanied by massive chromosomal aberrations. Upon orthotopic transplantation into a syngenic rat model, the recipient animals developed glioblastoma-like tumors. Molecular genetic analysis revealed a failure to downregulate the PDGF receptor alpha in transformed cell lines. A knock-down of the receptor by small interfering RNA molecules, as well as a PDGF receptor specific receptor tyrosine kinase inhibitor significantly inhibited cell growth in the transformed cell lines. PDGF is produced by normal as well as transformed precursor cell cultures. Hence, a brain tumor progression model is conceptualized in which normal CNS stem cells are converted into cancer stem cells by continued expression of PDGF receptor alpha, which would normally be downregulated upon differentiation. As this appears already after short intervals in culture, surveillance of neural stem/progenitor cultures for a potential transformation is mandatory before grafting.

Zusammenfassung

Die Entdeckung adulter Stammzellen im Gehirn von Säugetieren begründete die Forschung nach neurorekonstruktiven Therapieansätzen, welche sich jene Stammzellen zu Nutze machen. Erste Studien konnten zeigen, dass die Transplantation adulter Stammzellen für die Therapie neurodegenerativer Erkrankungen förderlich sein kann. Bevor diese eindrucksvollen Ergebnisse in präklinischen Studien angewandt werden können, sind eine Reihe von Fragen zu klären. Untersuchungen mit embryonalen Stammzellen zeigten, dass die aussichtsreichste Strategie für rekonstruktiven Zellersatz die Transplantation von bereits vor-differenzierten neuralen Vorläuferzellen ist. Diese integrieren sich funk-

tionell in das Gehirn des Transplantatempfängers und reifen entlang der ihnen vorgegebenen zellulären Differenzierungslinie. Effiziente Protokolle für die Differenzierung adulter Stammzellen des ZNS sind allerdings nur in geringem Umfang bekannt. Weiterhin haben sich bisher nur sehr wenige Arbeitsgruppen mit der Frage befasst, die Differenzier- und Transplantierbarkeit humaner adulter neuraler Stammzellen systematisch zu untersuchen. Experimente mit embryonalen Stammzellen haben allerdings ergeben, dass undifferenzierte ES-Zellen nach Transplantation Tumore erzeugen können – selbst wenn nur wenige dieser Zellen das Transplantat verunreinigen. Adulte Stammzellen gelten als entartungsresistent, jedoch müssen auch diese Zellen in Kultur vermehrt werden, um genügend Material für eine Transplantation zu erzeugen. Das Entartungspotenzial adulter Stammzellen des Gehirns *in vitro* wurde bisher allerdings noch nicht untersucht.

In der vorliegenden Arbeit wurde zunächst ein Protokoll für die neuronale Differenzierung adulter ZNS Vorläuferzellen aus Nagetieren etabliert. Durch den Einsatz von neuartigen Histon Deacetylase Inhibitoren gelang es, die Menge differenzierender Neurone zu verfünffachen. Die gebildeten Nervenzellen differenzierten in GABAerge und dopaminerge Zellen; die Ausbeute beider Zellpopulationen konnte merklich gesteigert werden. Im Gegensatz dazu nahm die oligodendrogliale Population um die Hälfte ab, während sich die Astrozytenzahl nicht signifikant veränderte.

Die molekularen Ursachen der Differenzierungsinduktion liegen in der Expressionssteigerung von pro-neuronalen Genen wie NeuroD1 und Cyclin D2, während die Expression oligodendroglialer Transkriptionsfaktoren (Olig2) abnimmt. Histon Deacetylase Inhibitoren scheinen daher in der Lage, die Differenzierung neuraler Vorläuferzellen von der oligodendroglialen Linie in die neuronale Linie umzulenken. Tatsächlich gibt es einen Vorläuferzelltyp *in vivo*, der beide Subpopulationen hervorbringen kann.

Das Protokoll für die Induktion neuronaler Differenzierung wurde anschließend auf humane hippocampale Vorläuferzellen übertragen. Humane Hippocampi wurden aus Epilepsie-chirurgischen Resektaten sowie aus Autopsiegewebe gewonnen. Während der Expansion exprimierten die Zellen Marker von Stamm- und Vorläuferzellen, wie z.B. Nestin und Musashi1. Marker von migrierenden Neuroblasten konnten nicht nachgewiesen werden, woraus sich ableiten lässt, dass es sich bei den isolierten Zellen um frühe neurale Vorläufer, sog. „*transit-amplifiers*“ handelt. Für die Differenzierung humaner Zellen wurden insgesamt drei verschiedene Protokolle getestet, von denen zwei bereits publiziert wurden. Eines der publizierten Protokolle und das neu etablierte Protokoll erlaubten die Induktion neuronaler Morphologie. Allerdings nahm die Zellzahl bei längerer Einwirkzeit nach dem publizierten Protokoll deutlich ab, während die Zellen bei kürzerer Behandlungsdauer wieder in den undifferenzierten Status zurückfielen. Dagegen ergab das neu etablierte Protokoll die solide Differenzierung in Nervenzellen, welche verschiedene Marker junger und reifer Neurone exprimierten. Insgesamt waren bis zu 70 % der induzierten Zellen für den neuronalen Marker Map2 positiv, während sich bis zu 6,4 % der Zellen für NeuN, ein Protein das ausschließlich von ausgereiften Nervenzellen gebildet wird, anfärben ließen. Zellkulturen von Patienten mit Ammonshornsklerose wurden mit Kulturen von Patienten, die keine AHS hatten, verglichen. Dabei ergab sich eine deutlich verminderte neuronale Differenzierungskapazität der Vorläuferzellen von AHS–Patienten. Da die Kulturzahlen noch gering sind, lässt sich daraus bislang keine verlässliche Aussage über die Zusammenhänge zwischen der Differenzierungsfähigkeit hippocampaler Vorläuferzellen und diesen Erkrankungstypen treffen.

Schließlich wurde die Frage adressiert, inwieweit adulte Vorläuferzellen *in vitro* entarten können und ob eine Entartung Konsequenzen für die Transplantation nach sich zieht. Adulte Vorläuferzellkulturen aus Ratten veränderten in Zellkultur bereits nach weniger als zehn Passagen ihren Phänotyp, was von massiven

chromosomalen Aberrationen begleitet wurde. Nach orthotoper Transplantation in ein syngenes Rattenmodell entwickelten die transplantierten Tiere glioblastom-ähnliche Tumoren. Molekulargenetische Analysen zeigten eine kontinuierliche Expression des PDGF Rezeptors alpha in den entarteten Zelllinien. Durch *knock-down* des Rezeptors mit siRNA-Molekülen, sowie durch einen spezifischen PDGF-Rezeptor-Tyrosinkinase Inhibitor konnte das Wachstum der entarteten Zelllinien signifikant gehemmt werden. PDGF wird von normalen sowie entarteten ZNS-Vorläuferzellen *in vitro* gebildet. Daraus ergibt sich folgendes Tumorentstehungsmodell: Stammzellen des ZNS exprimieren den PDGF Rezeptor alpha, regulieren diesen aber bei Differenzierung herab. Wird der Rezeptor allerdings weiterhin exprimiert, entarten die Zellen zu Tumorstammzellen. Da eine Entartung bereits nach kurzer Zeit in Zellkultur auftritt, ergibt sich die Notwendigkeit, neurale Vorläuferkulturen hinsichtlich einer möglichen Transformation zu überprüfen, bevor sie einer Transplantation zugeführt werden.

References

1. Allen, E. (1912) The Cessation of Mitosis in the Central Nervous System of the Albino Rat. *J Comp Neurol,* **22,** 547-568.
2. Altman, J. (1962) Are new neurons formed in the brains of adult mammals? *Science,* **135,** 1127-8.
3. Reynolds, B.A. and Weiss, S. (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science,* **255,** 1707-10.
4. Gross, C.G. (2000) Neurogenesis in the adult brain: death of a dogma. *Nat Rev Neurosci,* **1,** 67-73.
5. Kempermann, G. (2006) *Adult Neurogenesis*. Oxford University Press, Oxford.
6. Temple, S. (2006) Neural Stem Cells in Nervous System Development. In Rao, M.S. (ed.), *Neural Development and Stem Cells*. Humana Press, Inc., Totowa, NJ, USA, pp. 1-28.
7. Lie, D.C., Song, H., Colamarino, S.A., Ming, G.L. and Gage, F.H. (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. *Annu Rev Pharmacol Toxicol,* **44,** 399-421.
8. Gage, F.H. (2000) Mammalian neural stem cells. *Science,* **287,** 1433-8.
9. Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D. and Alvarez-Buylla, A. (2006) Origin of oligodendrocytes in the subventricular zone of the adult brain. *J Neurosci,* **26,** 7907-18.
10. Kosodo, Y., Roper, K., Haubensak, W., Marzesco, A.M., Corbeil, D. and Huttner, W.B. (2004) Asymmetric distribution of the apical plasma membrane during neurogenic divisions of mammalian neuroepithelial cells. *Embo J,* **23,** 2314-24.
11. Maslov, A.Y., Barone, T.A., Plunkett, R.J. and Pruitt, S.C. (2004) Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. *J Neurosci,* **24,** 1726-33.
12. Goldman, S.A., Kirschenbaum, B., Harrison-Restelli, C. and Thaler, H.T. (1997) Neuronal precursors of the adult rat subependymal zone persist into senescence, with no decline in spatial extent or response to BDNF. *J Neurobiol,* **32,** 554-66.
13. Alvarez-Buylla, A. and Lim, D.A. (2004) For the long run: maintaining germinal niches in the adult brain. *Neuron,* **41,** 683-6.
14. Alvarez-Buylla, A., Garcia-Verdugo, J.M. and Tramontin, A.D. (2001) A unified hypothesis on the lineage of neural stem cells. *Nat Rev Neurosci,* **2,** 287-93.
15. Heins, N., Malatesta, P., Cecconi, F., Nakafuku, M., Tucker, K.L., Hack, M.A., Chapouton, P., Barde, Y.A. and Gotz, M. (2002) Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci,* **5,** 308-15.
16. Merkle, F.T., Tramontin, A.D., Garcia-Verdugo, J.M. and Alvarez-Buylla, A. (2004) Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc Natl Acad Sci U S A.*
17. Anthony, T.E., Klein, C., Fishell, G. and Heintz, N. (2004) Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron,* **41,** 881-90.

18. Rakic, P. (1995) Radial versus tangential migration of neuronal clones in the developing cerebral cortex. *Proc Natl Acad Sci U S A*, **92,** 11323-7.
19. Lee, A., Kessler, J.D., Read, T.A., Kaiser, C., Corbeil, D., Huttner, W.B., Johnson, J.E. and Wechsler-Reya, R.J. (2005) Isolation of neural stem cells from the postnatal cerebellum. *Nat Neurosci*, **8,** 723-9.
20. Laywell, E.D., Kukekov, V.G. and Steindler, D.A. (1999) Multipotent neurospheres can be derived from forebrain subependymal zone and spinal cord of adult mice after protracted postmortem intervals. *Exp Neurol*, **156,** 430-3.
21. Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. and Gage, F.H. (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci*, **19,** 8487-97.
22. Lie, D.C., Dziewczapolski, G., Willhoite, A.R., Kaspar, B.K., Shults, C.W. and Gage, F.H. (2002) The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci*, **22,** 6639-49.
23. Ming, G.L. and Song, H. (2005) Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci*, **28,** 223-50.
24. Gould, E., Reeves, A.J., Graziano, M.S. and Gross, C.G. (1999) Neurogenesis in the neocortex of adult primates. *Science*, **286,** 548-52.
25. Zhao, M., Momma, S., Delfani, K., Carlen, M., Cassidy, R.M., Johansson, C.B., Brismar, H., Shupliakov, O., Frisen, J. and Janson, A.M. (2003) Evidence for neurogenesis in the adult mammalian substantia nigra. *Proc Natl Acad Sci U S A*, **100,** 7925-30.
26. Van Kampen, J.M., Hagg, T. and Robertson, H.A. (2004) Induction of neurogenesis in the adult rat subventricular zone and neostriatum following dopamine D receptor stimulation. *Eur J Neurosci*, **19,** 2377-87.
27. Cameron, H.A. and McKay, R.D. (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol*, **435,** 406-17.
28. Lemasson, M., Saghatelyan, A., Olivo-Marin, J.C. and Lledo, P.M. (2005) Neonatal and adult neurogenesis provide two distinct populations of newborn neurons to the mouse olfactory bulb. *J Neurosci*, **25,** 6816-25.
29. Doetsch, F., Garcia-Verdugo, J.M. and Alvarez-Buylla, A. (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci*, **17,** 5046-61.
30. Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M. and Alvarez-Buylla, A. (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*, **97,** 703-16.
31. Alvarez-Buylla, A. and Garcia-Verdugo, J.M. (2002) Neurogenesis in adult subventricular zone. *J Neurosci*, **22,** 629-34.
32. Doetsch, F. and Alvarez-Buylla, A. (1996) Network of tangential pathways for neuronal migration in adult mammalian brain. *Proc Natl Acad Sci U S A*, **93,** 14895-900.
33. Sanai, N., Tramontin, A.D., Quinones-Hinojosa, A., Barbaro, N.M., Gupta, N., Kunwar, S., Lawton, M.T., McDermott, M.W., Parsa, A.T., Manuel-Garcia Verdugo, J. *et al.* (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature*, **427,** 740-4.
34. Curtis, M.A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holtas, S., van Roon-Mom, W.M., Bjork-Eriksson, T., Nordborg, C. *et al.* (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science*, **315,** 1243-9.

35. Seaberg, R.M. and van der Kooy, D. (2002) Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci,* **22,** 1784-93.
36. Goldman, S. (2005) Stem and progenitor cell-based therapy of the human central nervous system. *Nat Biotechnol,* **23,** 862-71.
37. Bull, N.D. and Bartlett, P.F. (2005) The adult mouse hippocampal progenitor is neurogenic but not a stem cell. *J Neurosci,* **25,** 10815-21.
38. Seri, B., Garcia-Verdugo, J.M., McEwen, B.S. and Alvarez-Buylla, A. (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci,* **21,** 7153-60.
39. Seri, B., Garcia-Verdugo, J.M., Collado-Morente, L., McEwen, B.S. and Alvarez-Buylla, A. (2004) Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. *J Comp Neurol,* **478,** 359-78.
40. Palmer, T.D., Willhoite, A.R. and Gage, F.H. (2000) Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol,* **425,** 479-94.
41. Fabel, K., Tam, B., Kaufer, D., Baiker, A., Simmons, N., Kuo, C.J. and Palmer, T.D. (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *Eur J Neurosci,* **18,** 2803-12.
42. Jin, K., Zhu, Y., Sun, Y., Mao, X.O., Xie, L. and Greenberg, D.A. (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A,* **99,** 11946-50.
43. Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A. and Gage, F.H. (1998) Neurogenesis in the adult human hippocampus. *Nat Med,* **4,** 1313-7.
44. Goldman, S.A. and Nottebohm, F. (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proc Natl Acad Sci U S A,* **80,** 2390-4.
45. Byrd, C.A. and Brunjes, P.C. (2001) Neurogenesis in the olfactory bulb of adult zebrafish. *Neuroscience,* **105,** 793-801.
46. Amrein, I., Dechmann, D.K., Winter, Y. and Lipp, H.P. (2007) Absent or low rate of adult neurogenesis in the hippocampus of bats (chiroptera). *PLoS ONE,* **2,** e455.
47. Kempermann, G., Kuhn, H.G. and Gage, F.H. (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature,* **386,** 493-5.
48. Kempermann, G., Wiskott, L. and Gage, F.H. (2004) Functional significance of adult neurogenesis. *Curr Opin Neurobiol,* **14,** 186-91.
49. Meshi, D., Drew, M.R., Saxe, M., Ansorge, M.S., David, D., Santarelli, L., Malapani, C., Moore, H. and Hen, R. (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nat Neurosci,* **9,** 729-31.
50. Merrill, D.A., Karim, R., Darraq, M., Chiba, A.A. and Tuszynski, M.H. (2003) Hippocampal cell genesis does not correlate with spatial learning ability in aged rats. *J Comp Neurol,* **459,** 201-7.
51. Shors, T.J., Townsend, D.A., Zhao, M., Kozorovitskiy, Y. and Gould, E. (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus,* **12,** 578-84.
52. Brown, J., Cooper-Kuhn, C.M., Kempermann, G., Van Praag, H., Winkler, J., Gage, F.H. and Kuhn, H.G. (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci,* **17,** 2042-6.

53. Rochefort, C., Gheusi, G., Vincent, J.D. and Lledo, P.M. (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. *J Neurosci*, **22,** 2679-89.
54. Emsley, J.G., Mitchell, B.D., Kempermann, G. and Macklis, J.D. (2005) Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol*, **75,** 321-41.
55. Maguire, E.A., Gadian, D.G., Johnsrude, I.S., Good, C.D., Ashburner, J., Frackowiak, R.S. and Frith, C.D. (2000) Navigation-related structural change in the hippocampi of taxi drivers. *Proc Natl Acad Sci U S A*, **97,** 4398-403.
56. Hoglinger, G.U., Rizk, P., Muriel, M.P., Duyckaerts, C., Oertel, W.H., Caille, I. and Hirsch, E.C. (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. *Nat Neurosci*, **7,** 726-35.
57. Braak, H., Ghebremedhin, E., Rub, U., Bratzke, H. and Del Tredici, K. (2004) Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res*, **318,** 121-34.
58. Sobel, N., Thomason, M.E., Stappen, I., Tanner, C.M., Tetrud, J.W., Bower, J.M., Sullivan, E.V. and Gabrieli, J.D. (2001) An impairment in sniffing contributes to the olfactory impairment in Parkinson's disease. *Proc Natl Acad Sci U S A*, **98,** 4154-9.
59. Berry, M., Butt, A.M., Wilkin, G. and Perry, V.H. (2002) Structure and function of glia in the central nervous system. In Graham, D.I. and Lantos, P.L. (eds.), *Greenfield´s Neuropathology*. 7 ed. Arnold, London, Vol. 1, pp. 75-121.
60. Spassky, N., Merkle, F.T., Flames, N., Tramontin, A.D., Garcia-Verdugo, J.M. and Alvarez-Buylla, A. (2005) Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J Neurosci*, **25,** 10-8.
61. Pfenninger, C.V., Roschupkina, T., Hertwig, F., Kottwitz, D., Englund, E., Bengzon, J., Jacobsen, S.E. and Nuber, U.A. (2007) CD133 Is Not Present on Neurogenic Astrocytes in the Adult Subventricular Zone, but on Embryonic Neural Stem Cells, Ependymal Cells, and Glioblastoma Cells. *Cancer Res*, **67,** 5727-36.
62. Winner, B., Cooper-Kuhn, C.M., Aigner, R., Winkler, J. and Kuhn, H.G. (2002) Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *Eur J Neurosci*, **16,** 1681-9.
63. Santa-Olalla, J., Baizabal, J.M., Fregoso, M., del Carmen Cardenas, M. and Covarrubias, L. (2003) The in vivo positional identity gene expression code is not preserved in neural stem cells grown in culture. *Eur J Neurosci*, **18,** 1073-84.
64. Suhonen, J.O., Peterson, D.A., Ray, J. and Gage, F.H. (1996) Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature*, **383,** 624-7.
65. Christie, B.R. and Cameron, H.A. (2006) Neurogenesis in the adult hippocampus. *Hippocampus*, **16,** 199-207.
66. Bedard, A. and Parent, A. (2004) Evidence of newly generated neurons in the human olfactory bulb. *Brain Res Dev Brain Res*, **151,** 159-68.
67. Brown, J.P., Couillard-Despres, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L. and Kuhn, H.G. (2003) Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol*, **467,** 1-10.
68. Whittemore, S.R., Morassutti, D.J., Walters, W.M., Liu, R.H. and Magnuson, D.S. (1999) Mitogen and substrate differentially affect the lineage restriction of

adult rat subventricular zone neural precursor cell populations. *Exp Cell Res,* **252,** 75-95.
69. Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., 3rd and Nusse, R. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature,* **423,** 448-52.
70. Muroyama, Y., Kondoh, H. and Takada, S. (2004) Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochem Biophys Res Commun,* **313,** 915-21.
71. Lie, D.C., Colamarino, S.A., Song, H.J., Desire, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R. *et al.* (2005) Wnt signalling regulates adult hippocampal neurogenesis. *Nature,* **437,** 1370-5.
72. Irvin, D.K., Dhaka, A., Hicks, C., Weinmaster, G. and Kornblum, H.I. (2003) Extrinsic and intrinsic factors governing cell fate in cortical progenitor cultures. *Dev Neurosci,* **25,** 162-72.
73. Garcion, E., Halilagic, A., Faissner, A. and ffrench-Constant, C. (2004) Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. *Development,* **131,** 3423-32.
74. Jones, L.S. and Grooms, S.Y. (1997) Normal and aberrant functions of integrins in the adult central nervous system. *Neurochem Int,* **31,** 587-95.
75. Nakamoto, M. (2000) Eph receptors and ephrins. *Int J Biochem Cell Biol,* **32,** 7-12.
76. Schmucker, D. and Zipursky, S.L. (2001) Signaling downstream of Eph receptors and ephrin ligands. *Cell,* **105,** 701-4.
77. Hsieh, J. and Gage, F.H. (2004) Epigenetic control of neural stem cell fate. *Curr Opin Genet Dev,* **14,** 461-9.
78. Ajamian, F., Suuronen, T., Salminen, A. and Reeben, M. (2003) Upregulation of class II histone deacetylases mRNA during neural differentiation of cultured rat hippocampal progenitor cells. *Neurosci Lett,* **346,** 57-60.
79. Liu, A., Muggironi, M., Marin-Husstege, M. and Casaccia-Bonnefil, P. (2003) Oligodendrocyte process outgrowth in vitro is modulated by epigenetic regulation of cytoskeletal severing proteins. *Glia,* **44,** 264-74.
80. Marin-Husstege, M., Muggironi, M., Liu, A. and Casaccia-Bonnefil, P. (2002) Histone deacetylase activity is necessary for oligodendrocyte lineage progression. *J Neurosci,* **22,** 10333-45.
81. Hsieh, J., Nakashima, K., Kuwabara, T., Mejia, E. and Gage, F.H. (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A,* **101,** 16659-64.
82. Laeng, P., Pitts, R.L., Lemire, A.L., Drabik, C.E., Weiner, A., Tang, H., Thyagarajan, R., Mallon, B.S. and Altar, C.A. (2004) The mood stabilizer valproic acid stimulates GABA neurogenesis from rat forebrain stem cells. *J Neurochem,* **91,** 238-51.
83. Reik, W. and Dean, W. (2003) Mammalian Epigenomics: Reprogramming the Genome for Development and Therapy. In Beck, S. and Olek, A. (eds.), *The Epigenome - Molecular Hide and Seek.* Wiley-VCh, Weinheim, pp. 67 - 78.
84. Ferguson-Smith, A. (2003) At the Controls: Genomic Imprinting and the Epigenetic Regulation of Gene Expression. In Beck, S. and Olek, A. (eds.), *The Epigenome - Molecular Hide and Seek.* Wiley-VCh, Weinheim, pp. 81 - 99.
85. Millar, D.S., Holliday, R. and Grigg, G.W. (2003) Five not Four: History and Significance of the Fifth Base. In Beck, S. and Olek, A. (eds.), *The Epigenome - Molecular Hide and Seek.* Wiley-VCh, Weinheim, pp. 3 - 20.

86. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science*, **293,** 1074-80.
87. Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature*, **403,** 41-5.
88. Peterson, C.L. and Laniel, M.A. (2004) Histones and histone modifications. *Curr Biol*, **14,** R546-51.
89. Pradhan, S., Bacolla, A., Wells, R.D. and Roberts, R.J. (1999) Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem*, **274,** 33002-10.
90. de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S. and van Kuilenburg, A.B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J*, **370,** 737-49.
91. Marks, P.A., Miller, T. and Richon, V.M. (2003) Histone deacetylases. *Curr Opin Pharmacol*, **3,** 344-51.
92. Marks, P.A., Richon, V.M., Breslow, R. and Rifkind, R.A. (2001) Histone deacetylase inhibitors as new cancer drugs. *Curr Opin Oncol*, **13,** 477-83.
93. McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2001) Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev*, **11,** 497-504.
94. McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2002) Signaling chromatin to make muscle. *Curr Opin Cell Biol*, **14,** 763-72.
95. Richon, V.M., Sandhoff, T.W., Rifkind, R.A. and Marks, P.A. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A*, **97,** 10014-9.
96. Naruse, Y., Aoki, T., Kojima, T. and Mori, N. (1999) Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. *Proc Natl Acad Sci U S A*, **96,** 13691-6.
97. Schoenherr, C.J. and Anderson, D.J. (1995) The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science*, **267,** 1360-3.
98. Kuwabara, T., Hsieh, J., Nakashima, K., Taira, K. and Gage, F.H. (2004) A small modulatory dsRNA specifies the fate of adult neural stem cells. *Cell*, **116,** 779-93.
99. Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, Y., Nishino, N. and Horinouchi, S. (2001) Histone deacetylase as a new target for cancer chemotherapy. *Cancer Chemother Pharmacol*, **48 Suppl 1,** S20-6.
100. Merkle, F.T. and Alvarez-Buylla, A. (2006) Neural stem cells in mammalian development. *Curr Opin Cell Biol*, **18,** 704-9.
101. Laywell, E.D., Kearns, S.M., Zheng, T., Chen, K.A., Deng, J., Chen, H.X., Roper, S.N. and Steindler, D.A. (2005) Neuron-to-astrocyte transition: Phenotypic fluidity and the formation of hybrid asterons in differentiating neurospheres. *J Comp Neurol*, **493,** 321-33.
102. Imura, T., Kornblum, H.I. and Sofroniew, M.V. (2003) The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. *J Neurosci*, **23,** 2824-32.
103. Steindler, D.A. and Laywell, E.D. (2003) Astrocytes as stem cells: nomenclature, phenotype, and translation. *Glia*, **43,** 62-9.
104. Imura, T., Nakano, I., Kornblum, H.I. and Sofroniew, M.V. (2006) Phenotypic and functional heterogeneity of GFAP-expressing cells in vitro: differential

expression of LeX/CD15 by GFAP-expressing multipotent neural stem cells and non-neurogenic astrocytes. *Glia*, **53,** 277-93.
105. Galli, R., Gritti, A., Bonfanti, L. and Vescovi, A.L. (2003) Neural stem cells: an overview. *Circ Res*, **92,** 598-608.
106. Kempermann, G. and Kronenberg, G. (2003) Depressed new neurons--adult hippocampal neurogenesis and a cellular plasticity hypothesis of major depression. *Biol Psychiatry*, **54,** 499-503.
107. Jin, K., Galvan, V., Xie, L., Mao, X.O., Gorostiza, O.F., Bredesen, D.E. and Greenberg, D.A. (2004) Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPSw,Ind) mice. *Proc Natl Acad Sci U S A*, **101,** 13363-7.
108. Jessberger, S., Romer, B., Babu, H. and Kempermann, G. (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Exp Neurol*, **196,** 342-51.
109. Chevallier, N.L., Soriano, S., Kang, D.E., Masliah, E., Hu, G. and Koo, E.H. (2005) Perturbed neurogenesis in the adult hippocampus associated with presenilin-1 A246E mutation. *Am J Pathol*, **167,** 151-9.
110. Freundlieb, N., Francois, C., Tande, D., Oertel, W.H., Hirsch, E.C. and Hoglinger, G.U. (2006) Dopaminergic substantia nigra neurons project topographically organized to the subventricular zone and stimulate precursor cell proliferation in aged primates. *J Neurosci*, **26,** 2321-5.
111. Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N. and Ferriero, D.M. (2002) Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol*, **52,** 802-13.
112. Picard-Riera, N., Nait-Oumesmar, B. and Baron-Van Evercooren, A. (2004) Endogenous adult neural stem cells: limits and potential to repair the injured central nervous system. *J Neurosci Res*, **76,** 223-31.
113. Jin, K., Peel, A.L., Mao, X.O., Xie, L., Cottrell, B.A., Henshall, D.C. and Greenberg, D.A. (2004) Increased hippocampal neurogenesis in Alzheimer's disease. *Proc Natl Acad Sci U S A*, **101,** 343-7.
114. Parent, J.M. (2002) The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy Res*, **50,** 179-89.
115. Malberg, J.E., Eisch, A.J., Nestler, E.J. and Duman, R.S. (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci*, **20,** 9104-10.
116. Curtis, M.A., Penney, E.B., Pearson, A.G., van Roon-Mom, W.M., Butterworth, N.J., Dragunow, M., Connor, B. and Faull, R.L. (2003) Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain. *Proc Natl Acad Sci U S A*, **100,** 9023-7.
117. Jacobs, B.L., Praag, H. and Gage, F.H. (2000) Adult brain neurogenesis and psychiatry: a novel theory of depression. *Mol Psychiatry*, **5,** 262-9.
118. Encinas, J.M., Vaahtokari, A. and Enikolopov, G. (2006) Fluoxetine targets early progenitor cells in the adult brain. *Proc Natl Acad Sci U S A*, **103,** 8233-8.
119. Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O. *et al.* (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science*, **301,** 805-9.
120. Blumcke, I., Schewe, J.C., Normann, S., Brustle, O., Schramm, J., Elger, C.E. and Wiestler, O.D. (2001) Increase of nestin-immunoreactive neural precursor cells in the dentate gyrus of pediatric patients with early-onset temporal lobe epilepsy. *Hippocampus*, **11,** 311-21.

121. Heinrich, C., Nitta, N., Flubacher, A., Muller, M., Fahrner, A., Kirsch, M., Freiman, T., Suzuki, F., Depaulis, A., Frotscher, M. *et al.* (2006) Reelin deficiency and displacement of mature neurons, but not neurogenesis, underlie the formation of granule cell dispersion in the epileptic hippocampus. *J Neurosci,* **26,** 4701-13.
122. Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H. and Del Tredici, K. (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol (Berl),* **112,** 389-404.
123. Caille, I., Allinquant, B., Dupont, E., Bouillot, C., Langer, A., Muller, U. and Prochiantz, A. (2004) Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. *Development,* **131,** 2173-81.
124. Chen, Y. and Tang, B.L. (2006) The amyloid precursor protein and postnatal neurogenesis/neuroregeneration. *Biochem Biophys Res Commun,* **341,** 1-5.
125. Kwak, Y.D., Brannen, C.L., Qu, T., Kim, H.M., Dong, X., Soba, P., Majumdar, A., Kaplan, A., Beyreuther, K. and Sugaya, K. (2006) Amyloid precursor protein regulates differentiation of human neural stem cells. *Stem Cells Dev,* **15,** 381-9.
126. Lindvall, O., Kokaia, Z. and Martinez-Serrano, A. (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med,* **10 Suppl,** S42-50.
127. Lindvall, O. and Bjorklund, A. (2000) First step towards cell therapy for Huntington's disease. *Lancet,* **356,** 1945-6.
128. Lindvall, O. and Kokaia, Z. (2006) Stem cells for the treatment of neurological disorders. *Nature,* **441,** 1094-6.
129. Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., Ekdahl, C.T., Kokaia, Z. and Lindvall, O. (2006) Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells,* **24,** 739-47.
130. Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., Del Carro, U., Amadio, S., Bergami, A. *et al.* (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature,* **422,** 688-94.
131. Erdo, F., Buhrle, C., Blunk, J., Hoehn, M., Xia, Y., Fleischmann, B., Focking, M., Kustermann, E., Kolossov, E., Hescheler, J. *et al.* (2003) Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *J Cereb Blood Flow Metab,* **23,** 780-5.
132. Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E. and Scott, E.W. (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature,* **416,** 542-5.
133. Alvarez-Dolado, M., Pardal, R., Garcia-Verdugo, J.M., Fike, J.R., Lee, H.O., Pfeffer, K., Lois, C., Morrison, S.J. and Alvarez-Buylla, A. (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature,* **425,** 968-73.
134. Jordan, C.T., Guzman, M.L. and Noble, M. (2006) Cancer stem cells. *N Engl J Med,* **355,** 1253-61.
135. Reya, T., Morrison, S.J., Clarke, M.F. and Weissman, I.L. (2001) Stem cells, cancer, and cancer stem cells. *Nature,* **414,** 105-11.

136. Vescovi, A.L., Galli, R. and Reynolds, B.A. (2006) Brain tumour stem cells. *Nat Rev Cancer*, **6,** 425-36.
137. Al-Hajj, M. and Clarke, M.F. (2004) Self-renewal and solid tumor stem cells. *Oncogene*, **23,** 7274-82.
138. Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A. and Dick, J.E. (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, **367,** 645-8.
139. Ignatova, T.N., Kukekov, V.G., Laywell, E.D., Suslov, O.N., Vrionis, F.D. and Steindler, D.A. (2002) Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia*, **39,** 193-206.
140. Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J. and Dirks, P.B. (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res*, **63,** 5821-8.
141. Piccirillo, S.G., Reynolds, B.A., Zanetti, N., Lamorte, G., Binda, E., Broggi, G., Brem, H., Olivi, A., Dimeco, F. and Vescovi, A.L. (2006) Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature*, **444,** 761-5.
142. Collins, A.T., Berry, P.A., Hyde, C., Stower, M.J. and Maitland, N.J. (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*, **65,** 10946-51.
143. O'Brien, C.A., Pollett, A., Gallinger, S. and Dick, J.E. (2007) A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, **445,** 106-10.
144. Sanai, N., Alvarez-Buylla, A. and Berger, M.S. (2005) Neural stem cells and the origin of gliomas. *N Engl J Med*, **353,** 811-22.
145. Miura, M., Miura, Y., Padilla-Nash, H.M., Molinolo, A.A., Fu, B., Patel, V., Seo, B.M., Sonoyama, W., Zheng, J.J., Baker, C.C. *et al.* (2006) Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells*, **24,** 1095-103.
146. Rubio, D., Garcia-Castro, J., Martin, M.C., de la Fuente, R., Cigudosa, J.C., Lloyd, A.C. and Bernad, A. (2005) Spontaneous human adult stem cell transformation. *Cancer Res*, **65,** 3035-9.
147. Dai, C., Celestino, J.C., Okada, Y., Louis, D.N., Fuller, G.N. and Holland, E.C. (2001) PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. *Genes Dev*, **15,** 1913-25.
148. Jackson, E.L., Garcia-Verdugo, J.M., Gil-Perotin, S., Roy, M., Quinones-Hinojosa, A., VandenBerg, S. and Alvarez-Buylla, A. (2006) PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron*, **51,** 187-99.
149. Foroni, C., Galli, R., Cipelletti, B., Caumo, A., Alberti, S., Fiocco, R. and Vescovi, A. (2007) Resilience to Transformation and Inherent Genetic and Functional Stability of Adult Neural Stem Cells Ex vivo. *Cancer Res*, **67,** 3725-33.
150. Corbeil, D., Fargeas, C.A. and Huttner, W.B. (2001) Rat prominin, like its mouse and human orthologues, is a pentaspan membrane glycoprotein. *Biochem Biophys Res Commun*, **285,** 939-44.
151. Corbeil, D., Roper, K., Hannah, M.J., Hellwig, A. and Huttner, W.B. (1999) Selective localization of the polytopic membrane protein prominin in microvilli

of epithelial cells - a combination of apical sorting and retention in plasma membrane protrusions. *J Cell Sci*, **112 (Pt 7),** 1023-33.
152. Hockfield, S. and McKay, R.D. (1985) Identification of major cell classes in the developing mammalian nervous system. *J Neurosci*, **5,** 3310-28.
153. Holsken, A., Eyupoglu, I.Y., Lueders, M., Trankle, C., Dieckmann, D., Buslei, R., Hahnen, E., Blumcke, I. and Siebzehnrubl, F.A. (2006) Ex vivo therapy of malignant melanomas transplanted into organotypic brain slice cultures using inhibitors of histone deacetylases. *Acta Neuropathol (Berl)*, **112,** 205-15.
154. Buslei, R., Kreutzer, J., Hofmann, B., Schmidt, V., Siebzehnrubl, F., Hahnen, E., Eyupoglu, I.Y., Fahlbusch, R. and Blumcke, I. (2006) Abundant hypermethylation of SOCS-1 in clinically silent pituitary adenomas. *Acta Neuropathol (Berl)*, **111,** 264-71.
155. Brichta, L., Hofmann, Y., Hahnen, E., Siebzehnrubl, F.A., Raschke, H., Blumcke, I., Eyupoglu, I.Y. and Wirth, B. (2003) Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. *Hum Mol Genet*, **12,** 2481-9.
156. Goldstein, S. (1990) Replicative senescence: the human fibroblast comes of age. *Science*, **249,** 1129-33.
157. Paxinos, G. and Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, San Diego.
158. Vistica, D.T., Skehan, P., Scudiero, D., Monks, A., Pittman, A. and Boyd, M.R. (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production. *Cancer Res*, **51,** 2515-20.
159. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65,** 55-63.
160. Eyupoglu, I.Y., Hahnen, E., Buslei, R., Siebzehnrubl, F.A., Savaskan, N.E., Luders, M., Trankle, C., Wick, W., Weller, M., Fahlbusch, R. *et al.* (2005) Suberoylanilide hydroxamic acid (SAHA) has potent anti-glioma properties in vitro, ex vivo and in vivo. *J Neurochem*, **93,** 992-9.
161. Vescovi, A.L., Galli, R. and Gritti, A. (2002) Clonal Analyses and Cryopreservation of Neural Stem Cell Cultures. In Zigova, T., Sanberg, P.R. and Sanchez-Ramos, J.R. (eds.), *Neural Stem Cells - Methods and Protocols*. Humana Press, Totowa, NJ, Vol. 198, pp. 115 - 123.
162. Reynolds, B.A. and Rietze, R.L. (2005) Neural stem cells and neurospheres--re-evaluating the relationship. *Nat Methods*, **2,** 333-6.
163. Sommer, I. and Schachner, M. (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol*, **83,** 311-27.
164. Belachew, S., Yuan, X. and Gallo, V. (2001) Unraveling oligodendrocyte origin and function by cell-specific transgenesis. *Dev Neurosci*, **23,** 287-98.
165. Bignami, A., Eng, L.F., Dahl, D. and Uyeda, C.T. (1972) Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res*, **43,** 429-35.
166. Raponi, E., Agenes, F., Delphin, C., Assard, N., Baudier, J., Legraverend, C. and Deloulme, J.C. (2007) S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. *Glia*, **55,** 165-77.
167. Ferreira, A. and Caceres, A. (1992) Expression of the class III beta-tubulin isotype in developing neurons in culture. *J Neurosci Res*, **32,** 516-29.

168. Marks, P.A. and Jiang, X. (2005) Histone deacetylase inhibitors in programmed cell death and cancer therapy. *Cell Cycle*, **4,** 549-51.
169. Angelastro, J.M., Ignatova, T.N., Kukekov, V.G., Steindler, D.A., Stengren, G.B., Mendelsohn, C. and Greene, L.A. (2003) Regulated expression of ATF5 is required for the progression of neural progenitor cells to neurons. *J Neurosci*, **23,** 4590-600.
170. Mason, J.L., Angelastro, J.M., Ignatova, T.N., Kukekov, V.G., Lin, G., Greene, L.A. and Goldman, J.E. (2005) ATF5 regulates the proliferation and differentiation of oligodendrocytes. *Mol Cell Neurosci*, **29,** 372-80.
171. Tirone, F. (2001) The gene PC3(TIS21/BTG2), prototype member of the PC3/BTG/TOB family: regulator in control of cell growth, differentiation, and DNA repair? *J Cell Physiol*, **187,** 155-65.
172. Canzoniere, D., Farioli-Vecchioli, S., Conti, F., Ciotti, M.T., Tata, A.M., Augusti-Tocco, G., Mattei, E., Lakshmana, M.K., Krizhanovsky, V., Reeves, S.A. *et al*. (2004) Dual control of neurogenesis by PC3 through cell cycle inhibition and induction of Math1. *J Neurosci*, **24,** 3355-69.
173. Iacopetti, P., Michelini, M., Stuckmann, I., Oback, B., Aaku-Saraste, E. and Huttner, W.B. (1999) Expression of the antiproliferative gene TIS21 at the onset of neurogenesis identifies single neuroepithelial cells that switch from proliferative to neuron-generating division. *Proc Natl Acad Sci U S A*, **96,** 4639-44.
174. Johansson, C.B., Momma, S., Clarke, D.L., Risling, M., Lendahl, U. and Frisen, J. (1999) Identification of a neural stem cell in the adult mammalian central nervous system. *Cell*, **96,** 25-34.
175. Walton, N.M., Sutter, B.M., Chen, H.X., Chang, L.J., Roper, S.N., Scheffler, B. and Steindler, D.A. (2006) Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. *Development*, **133,** 3671-81.
176. Milosevic, J., Brandt, A., Roemuss, U., Arnold, A., Wegner, F., Schwarz, S.C., Storch, A., Zimmermann, H. and Schwarz, J. (2006) Uracil nucleotides stimulate human neural precursor cell proliferation and dopaminergic differentiation: involvement of MEK/ERK signalling. *J Neurochem*, **99,** 913-23.
177. Blaess, S., Corrales, J.D. and Joyner, A.L. (2006) Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. *Development*, **133,** 1799-809.
178. Siebzehnrubl, F.A., Buslei, R., Eyupoglu, I.Y., Seufert, S., Hahnen, E. and Blumcke, I. (2007) Histone deacetylase inhibitors increase neuronal differentiation in adult forebrain precursor cells. *Exp Brain Res*, **176,** 672-8.
179. Kim, M. and Morshead, C.M. (2003) Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J Neurosci*, **23,** 10703-9.
180. Hirschmann-Jax, C., Foster, A.E., Wulf, G.G., Nuchtern, J.G., Jax, T.W., Gobel, U., Goodell, M.A. and Brenner, M.K. (2004) A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A*, **101,** 14228-33.
181. Schenka, A.A., Machado, C.M., Grippo, M.C., Queiroz, L.S., Schenka, N.G., Chagas, C.A., Verinaud, L., Brousset, P. and Vassallo, J. (2005) Immunophenotypic and ultrastructural validation of a new human glioblastoma cell line. *Cell Mol Neurobiol*, **25,** 929-41.

182. Stojiljkovic, M., Piperski, V., Dacevic, M., Rakic, L., Ruzdijic, S. and Kanazir, S. (2003) Characterization of 9L glioma model of the Wistar rat. *J Neurooncol*, **63,** 1-7.
183. Giordana, M.T., Migheli, A., Mocellini, C., Villare, F. and Schiffer, D. (1992) Immunohistochemical observations on rat radial glia: relationship with the origin of ethylnitrosourea-induced tumors. *Acta Neuropathol (Berl)*, **84,** 387-93.
184. Parati, E.A., Bez, A., Ponti, D., Sala, S., Pozzi, S. and Pagano, S.F. (2003) Neural stem cells. Biological features and therapeutic potential in Parkinson's disease. *J Neurosurg Sci*, **47,** 8-17.
185. Gangemi, R.M., Perera, M. and Corte, G. (2004) Regulatory genes controlling cell fate choice in embryonic and adult neural stem cells. *J Neurochem*, **89,** 286-306.
186. de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J. *et al.* (1997) Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development*, **124,** 1139-48.
187. Rajan, P., Panchision, D.M., Newell, L.F. and McKay, R.D. (2003) BMPs signal alternately through a SMAD or FRAP-STAT pathway to regulate fate choice in CNS stem cells. *J Cell Biol*, **161,** 911-21.
188. Cui, X.Y., Hu, Q.D., Tekaya, M., Shimoda, Y., Ang, B.T., Nie, D.Y., Sun, L., Hu, W.P., Karsak, M., Duka, T. *et al.* (2004) NB-3/Notch1 pathway via Deltex1 promotes neural progenitor cell differentiation into oligodendrocytes. *J Biol Chem*, **279,** 25858-65.
189. Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D. and McKay, R.D. (1999) Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science*, **285,** 754-6.
190. Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A. and Ben-Hur, T. (2001) Neural progenitors from human embryonic stem cells. *Nat Biotechnol*, **19,** 1134-40.
191. Kukekov, V.G., Laywell, E.D., Suslov, O., Davies, K., Scheffler, B., Thomas, L.B., O'Brien, T.F., Kusakabe, M. and Steindler, D.A. (1999) Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp Neurol*, **156,** 333-44.
192. Kukekov, V.G., Laywell, E.D., Thomas, L.B. and Steindler, D.A. (1997) A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia*, **21,** 399-407.
193. Biron, V.L., McManus, K.J., Hu, N., Hendzel, M.J. and Underhill, D.A. (2004) Distinct dynamics and distribution of histone methyl-lysine derivatives in mouse development. *Dev Biol*, **276,** 337-51.
194. Jaenisch, R. and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*, **33 Suppl,** 245-54.
195. Hsieh, J. and Gage, F.H. (2005) Chromatin remodeling in neural development and plasticity. *Curr Opin Cell Biol*, **17,** 664-71.
196. Laywell, E.D., Rakic, P., Kukekov, V.G., Holland, E.C. and Steindler, D.A. (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc Natl Acad Sci U S A*, **97,** 13883-8.

197. Gould, E., Reeves, A.J., Fallah, M., Tanapat, P., Gross, C.G. and Fuchs, E. (1999) Hippocampal neurogenesis in adult Old World primates. *Proc Natl Acad Sci U S A*, **96,** 5263-7.
198. Miller, T.A., Witter, D.J. and Belvedere, S. (2003) Histone deacetylase inhibitors. *J Med Chem*, **46,** 5097-116.
199. Zhang, X.D., Gillespie, S.K., Borrow, J.M. and Hersey, P. (2003) The histone deacetylase inhibitor suberic bishydroxamate: a potential sensitizer of melanoma to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. *Biochem Pharmacol*, **66,** 1537-45.
200. Coyle, J.T. and Duman, R.S. (2003) Finding the intracellular signaling pathways affected by mood disorder treatments. *Neuron*, **38,** 157-60.
201. Jansen, M.S., Nagel, S.C., Miranda, P.J., Lobenhofer, E.K., Afshari, C.A. and McDonnell, D.P. (2004) Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. *Proc Natl Acad Sci U S A*, **101,** 7199-204.
202. Gurvich, N. and Klein, P.S. (2002) Lithium and valproic acid: parallels and contrasts in diverse signaling contexts. *Pharmacol Ther*, **96,** 45-66.
203. Gurvich, N., Tsygankova, O.M., Meinkoth, J.L. and Klein, P.S. (2004) Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res*, **64,** 1079-86.
204. Gottlicher, M., Minucci, S., Zhu, P., Kramer, O.H., Schimpf, A., Giavara, S., Sleeman, J.P., Lo Coco, F., Nervi, C., Pelicci, P.G. *et al.* (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *Embo J*, **20,** 6969-78.
205. Hahnen, E., Eyupoglu, I.Y., Brichta, L., Haastert, K., Trankle, C., Siebzehnrubl, F.A., Riessland, M., Holker, I., Claus, P., Romstock, J. *et al.* (2006) In vitro and ex vivo evaluation of second generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy. *J Neurochem*, **98,** 193-202.
206. Phiel, C.J., Zhang, F., Huang, E.Y., Guenther, M.G., Lazar, M.A. and Klein, P.S. (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem*, **276,** 36734-41.
207. Lindberger, M., Tomson, T., Wallstedt, L. and Stahle, L. (2001) Distribution of valproate to subdural cerebrospinal fluid, subcutaneous extracellular fluid, and plasma in humans: a microdialysis study. *Epilepsia*, **42,** 256-61.
208. Marks, P.A., Richon, V.M., Kelly, W.K., Chiao, J.H. and Miller, T. (2004) Histone deacetylase inhibitors: development as cancer therapy. *Novartis Found Symp*, **259,** 269-81; discussion 281-8.
209. Park, J.H., Jung, Y., Kim, T.Y., Kim, S.G., Jong, H.S., Lee, J.W., Kim, D.K., Lee, J.S., Kim, N.K. and Bang, Y.J. (2004) Class I histone deacetylase-selective novel synthetic inhibitors potently inhibit human tumor proliferation. *Clin Cancer Res*, **10,** 5271-81.
210. Kim, S.M., Yang, J.W., Park, M.J., Lee, J.K., Kim, S.U., Lee, Y.S. and Lee, M.A. (2006) Regulation of human tyrosine hydroxylase gene by neuron-restrictive silencer factor. *Biochem Biophys Res Commun*, **346,** 426-35.
211. Scheffler, B., Walton, N.M., Lin, D.D., Goetz, A.K., Enikolopov, G., Roper, S.N. and Steindler, D.A. (2005) Phenotypic and functional characterization of adult brain neuropoiesis. *Proc Natl Acad Sci U S A*, **102,** 9353-8.

212. Baker, G.L., Landis, M.W. and Hinds, P.W. (2005) Multiple functions of D-type cyclins can antagonize pRb-mediated suppression of proliferation. *Cell Cycle,* **4,** 330-8.
213. Sumrejkanchanakij, P., Tamamori-Adachi, M., Matsunaga, Y., Eto, K. and Ikeda, M.A. (2003) Role of cyclin D1 cytoplasmic sequestration in the survival of postmitotic neurons. *Oncogene,* **22,** 8723-30.
214. Kowalczyk, A., Filipkowski, R.K., Rylski, M., Wilczynski, G.M., Konopacki, F.A., Jaworski, J., Ciemerych, M.A., Sicinski, P. and Kaczmarek, L. (2004) The critical role of cyclin D2 in adult neurogenesis. *J Cell Biol,* **167,** 209-13.
215. Yoshida, Y., Matsuda, S., Ikematsu, N., Kawamura-Tsuzuku, J., Inazawa, J., Umemori, H. and Yamamoto, T. (1998) ANA, a novel member of Tob/BTG1 family, is expressed in the ventricular zone of the developing central nervous system. *Oncogene,* **16,** 2687-93.
216. Guardavaccaro, D., Corrente, G., Covone, F., Micheli, L., D'Agnano, I., Starace, G., Caruso, M. and Tirone, F. (2000) Arrest of G(1)-S progression by the p53-inducible gene PC3 is Rb dependent and relies on the inhibition of cyclin D1 transcription. *Mol Cell Biol,* **20,** 1797-815.
217. Ligon, K.L., Fancy, S.P., Franklin, R.J. and Rowitch, D.H. (2006) Olig gene function in CNS development and disease. *Glia,* **54,** 1-10.
218. Hack, M.A., Saghatelyan, A., de Chevigny, A., Pfeifer, A., Ashery-Padan, R., Lledo, P.M. and Gotz, M. (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat Neurosci,* **8,** 865-72.
219. Cunliffe, V.T. and Casaccia-Bonnefil, P. (2006) Histone deacetylase 1 is essential for oligodendrocyte specification in the zebrafish CNS. *Mech Dev,* **123,** 24-30.
220. Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H. and Weissman, I.L. (2000) Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A,* **97,** 14720-5.
221. Islam, M.O., Kanemura, Y., Tajria, J., Mori, H., Kobayashi, S., Hara, M., Yamasaki, M., Okano, H. and Miyake, J. (2005) Functional expression of ABCG2 transporter in human neural stem/progenitor cells. *Neurosci Res,* **52,** 75-82.
222. Tarasenko, Y.I., Yu, Y., Jordan, P.M., Bottenstein, J. and Wu, P. (2004) Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. *J Neurosci Res,* **78,** 625-36.
223. Kim, H.T., Kim, I.S., Lee, I.S., Lee, J.P., Snyder, E.Y. and Park, K.I. (2006) Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed. *Exp Neurol,* **199,** 222-35.
224. Cacci, E., Villa, A., Parmar, M., Cavallaro, M., Mandahl, N., Lindvall, O., Martinez-Serrano, A. and Kokaia, Z. (2006) Generation of human cortical neurons from a new immortal fetal neural stem cell line. *Exp Cell Res.*
225. Schwarz, J., Schwarz, S.C. and Storch, A. (2006) Developmental perspectives on human midbrain-derived neural stem cells. *Neurodegener Dis,* **3,** 45-9.
226. Suzuki, M., Nelson, A.D., Eickstaedt, J.B., Wallace, K., Wright, L.S. and Svendsen, C.N. (2006) Glutamate enhances proliferation and neurogenesis in human neural progenitor cell cultures derived from the fetal cortex. *Eur J Neurosci,* **24,** 645-53.

227. Palmer, T.D., Schwartz, P.H., Taupin, P., Kaspar, B., Stein, S.A. and Gage, F.H. (2001) Cell culture. Progenitor cells from human brain after death. *Nature*, **411,** 42-3.
228. Kempermann, G., Chesler, E.J., Lu, L., Williams, R.W. and Gage, F.H. (2006) Natural variation and genetic covariance in adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A*, **103,** 780-5.
229. Kempermann, G. and Gage, F.H. (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. *Brain Res Dev Brain Res*, **134,** 1-12.
230. Parent, J.M., von dem Bussche, N. and Lowenstein, D.H. (2006) Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. *Hippocampus*, **16,** 321-8.
231. Doetsch, F. (2003) The glial identity of neural stem cells. *Nat Neurosci*, **6,** 1127-34.
232. Blumcke, I., Pauli, E., Clusmann, H., Schramm, J., Becker, A., Elger, C., Merschhemke, M., Meencke, H.J., Lehmann, T., von Deimling, A. *et al.* (2007) A new clinico-pathological classification system for mesial temporal sclerosis. *Acta Neuropathol (Berl)*, **113,** 235-244.
233. Hinterkeuser, S., Schroder, W., Hager, G., Seifert, G., Blumcke, I., Elger, C.E., Schramm, J. and Steinhauser, C. (2000) Astrocytes in the hippocampus of patients with temporal lobe epilepsy display changes in potassium conductances. *Eur J Neurosci*, **12,** 2087-96.
234. Hsieh, J., Aimone, J.B., Kaspar, B.K., Kuwabara, T., Nakashima, K. and Gage, F.H. (2004) IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. *J Cell Biol*, **164,** 111-22.
235. Singh, S.K., Clarke, I.D., Hide, T. and Dirks, P.B. (2004) Cancer stem cells in nervous system tumors. *Oncogene*, **23,** 7267-73.
236. Doetsch, F. (2003) A niche for adult neural stem cells. *Curr Opin Genet Dev*, **13,** 543-50.
237. Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.-M. and Alvarez-Buylla, A. (2002) EGF Converts Transit-Amplifying Neurogenic Precursors in the Adult Brain into Multipotent Stem Cells. *Neuron*, **36,** 1021-1034.
238. Rangarajan, A., Hong, S.J., Gifford, A. and Weinberg, R.A. (2004) Species- and cell type-specific requirements for cellular transformation. *Cancer Cell*, **6,** 171-83.
239. Ligon, K.L., Huillard, E., Mehta, S., Kesari, S., Liu, H., Alberta, J.A., Bachoo, R.M., Kane, M., Louis, D.N., Depinho, R.A. *et al.* (2007) Olig2-regulated lineage-restricted pathway controls replication competence in neural stem cells and malignant glioma. *Neuron*, **53,** 503-17.
240. Kippin, T.E., Martens, D.J. and van der Kooy, D. (2005) p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev*, **19,** 756-67.
241. Mouthon, M.A., Fouchet, P., Mathieu, C., Sii-Felice, K., Etienne, O., Lages, C.S. and Boussin, F.D. (2006) Neural stem cells from mouse forebrain are contained in a population distinct from the 'side population'. *J Neurochem*, **99,** 807-17.
242. Hermanson, M., Funa, K., Koopmann, J., Maintz, D., Waha, A., Westermark, B., Heldin, C.H., Wiestler, O.D., Louis, D.N., von Deimling, A. *et al.* (1996) Association of loss of heterozygosity on chromosome 17p with high platelet-

derived growth factor alpha receptor expression in human malignant gliomas. *Cancer Res,* **56,** 164-71.

243. Smits, A., van Grieken, D., Hartman, M., Lendahl, U., Funa, K. and Nister, M. (1996) Coexpression of platelet-derived growth factor alpha and beta receptors on medulloblastomas and other primitive neuroectodermal tumors is consistent with an immature stem cell and neuronal derivation. *Lab Invest,* **74,** 188-98.

244. Lederle, W., Stark, H.J., Skobe, M., Fusenig, N.E. and Mueller, M.M. (2006) Platelet-derived growth factor-BB controls epithelial tumor phenotype by differential growth factor regulation in stromal cells. *Am J Pathol,* **169,** 1767-83.

245. Yeh, H.J., Ruit, K.G., Wang, Y.X., Parks, W.C., Snider, W.D. and Deuel, T.F. (1991) PDGF A-chain gene is expressed by mammalian neurons during development and in maturity. *Cell,* **64,** 209-16.

246. Sasahara, M., Fries, J.W., Raines, E.W., Gown, A.M., Westrum, L.E., Frosch, M.P., Bonthron, D.T., Ross, R. and Collins, T. (1991) PDGF B-chain in neurons of the central nervous system, posterior pituitary, and in a transgenic model. *Cell,* **64,** 217-27.

247. Chiasson, B.J., Tropepe, V., Morshead, C.M. and van der Kooy, D. (1999) Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J Neurosci,* **19,** 4462-71.

248. Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P.J., Aigner, L., Brawanski, A., Bogdahn, U. and Beier, C.P. (2007) CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res,* **67,** 4010-5.

249. Wernig, M., Benninger, F., Schmandt, T., Rade, M., Tucker, K.L., Bussow, H., Beck, H. and Brustle, O. (2004) Functional integration of embryonic stem cell-derived neurons in vivo. *J Neurosci,* **24,** 5258-68.

250. Lepore, A.C., Han, S.S., Tyler-Polsz, C.J., Cai, J., Rao, M.S. and Fischer, I. (2004) Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. *Neuron Glia Biol,* **1,** 113-126.

Appendix

List of abbreviations

A	Adenosine
ABCG2	ATP binding cassette transporter, subfamily G, member 2
AD	Alzheimer's disease
AHS	Ammon's horn sclerosis
ANOVA	Analysis of variance
APP	Amyloid precursor protein
Atf	Activating transcription factor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor (also: FGF2)
bHLH	Basic helix-loop-helix protein
BLBP	Brain lipid binding protein
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Btg	B-cell translocation gene
cm^2	Square centimetre
C	Cytosine
CA	Cornu ammonis
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CBHA	*m*-Carboxycinnamic acid bishydroxamate
CCD	Charge-coupled device
CD133	Cluster of differentiation 133 (also: Prominin 1)
CNPase	2',3'-cyclic nucleotide 3'-phospho-diesterase
CNS	Central nervous system
Co	Control
cP	Centipoise
CREB	cAMP response element binding protein
Dcx	Doublecortin
DIC	Days in culture
Dlx	Distal-less homeobox gene
DMEM	Dulbecco´s modified Eagle´s medium

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsRNA	double-stranded RNA
DTT	(*2S*,*3S*)-1,4-Bis-sulfanylbutane-2,3-diol (Dithiothreitol)
EDTA	Ethylenediamine-N,N-tetracetate
EGF	Epidermal growth factor
EGFR	EGF receptor
ES cell	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDA	Food and drug administration
FGF2	see bFGF
FSB	Fish skin gelatine buffer
g	Gravitational force
G	Guanine
GABA	Gamma-amino-butyric acid
GAD65/67	Glutamic acid decarboxylase 65/67 kDa
GalC	Galactosylceramide (also: galactocerebroside)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCD	Granule cell dispersion
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter 1
HAT	Histone acetyltransferase
HBSS	Hank's balanced salt solution
Hda	Yeast histone deacetylase
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IBMX	3-Isobutyl-1-methyl-xanthine
IC_{50}	Inhibitory concentration 50%
IC_{90}	Inhibitory concentration 90%
iHDAC	Inhibitor of histone deacetylases
Jak	Janus kinase
kDa	Kilodalton
LIF	Leukemia inhibitory factor
Lys	Lysine
M	Mol/Liter
µg	Microgram
mg	Milligram
µl	Microliter
ml	Milliliter
µM	Micromol/Liter
µm	Micrometer

mM	Millimol/Liter
mm	Millimeter
M344	4-Dimethylamino-N-(6-hydroxycarbamoyl-hexyl)-benzamide
Map2	Microtubuli associated protein 2
MBP	Myelin basic protein
min	Minutes
MMTV	Murine mammary tumor virus
MS275	*N*-(2-aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl)aminomethyl] benzamide
mSin3	murine homolog of yeast SIN3
MTLE	Mesial temporal lobe epilepsy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
ng	Nanogram
nM	Nanomol/Liter
nm	Nanometer
NAD^+	Nicotinamide adenosine dinucleotide (oxidized form)
NeuN	Neuronal nuclei
NeuroD	Neurogenic differentiation
NF-L	Neurofilament protein light chain
NGF	Nerve growth factor
NPC	Neural precursor cell
NRSF	Neuron restrictive silencing factor
O2A	Oligodendrocyte - type II astrocyte progenitor
O4	Oligodendrocyte surface glycoprotein
Olig	Oligodendrocyte lineage transcription factor
$p21^{WAF}$	Wildtype p53-activated fragment
p300	Adenovirus E1A associated protein of 300 kDa
p53	Tumor protein of 53 kDa
PBS	Phosphate buffered saline
PC3	Pheochromacytoma cell-3 (NGF-inducible early gene)
P/CAF	p300/CBP associating factor
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet-derived growth factor
PDGFRα	PDGF receptor α
PI	Propidium iodide
PFA	Paraformaldehyde
PMSF	Phenylmethanesulfonyl fluoride
PPD	Papain/Protease/DNase solution
PSA-NCAM	Polysialic acid neural cell adhesion molecule
PTEN	Phosphatase and tensin homolog
RE1	Response element 1
REST	RE1 silencing transcription factor

RMS	Rostral migratory stream
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
S	Svedberg units
SAHA	Suberoylanilide hydroxamic acid
SBHA	Suberoyl bishydroxamic acid (suberic bishydroxamate)
SD	Standard deviation
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean
siRNA	small interfering RNA
SGZ	Subgranular zone
Shh	Sonic hedgehog protein
Sir	Silent information regulator
SIRT	Sirtuin; also: Sir2-like
Sox	SRY-box protein
SPC	SVZ precursor cell
SRY	Sex determining region on Y chromosome
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
T	Thymidine
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
TH	Tyrosine hydroxylase
TLE	Temporal lobe epilepsy
TOB	Transducer of erbB2
Tris	Tris-(hydroxymethyl)-aminomethane
TSA	Trichostatin A
U	Uracil
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
vGlut1	Vesicular glutamate transporter 1
VPA	2- propylpentanoic acid (valproic acid)
Wnt	Wingless-type MMTV integration site
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β–D-galactopyranoside

Bibliography

Publications marked with an asterisk contain work that is described in this thesis.

(*) **Siebzehnrübl FA**, Jeske I, Müller D, Buslei R, Hahnen E, Huttner HB, Corbeil D, Kaesbauer J, Appl T, von Hörsten S, Blümcke I. Spontaneuos in vitro transformation of adult neural precursors into cancer stem cells. *Submitted*

Huttner HB, Janich P, Köhrmann M, Jászai J, **Siebzehnrübl FA**, Blümcke I, Suttorp M, Gahr M, Kuhnt D, Nimsky C, Krex D, Schackert G, Löwenbrück K, Reichmann H, Jüttler E, Hacke W, Schellinger PD, Schwab S, Marzesco AM, Corbeil D. The stem cell marker prominin-1/CD133 on membrane particles in human cerebrospinal fluid offers novel approaches for studying CNS disease. *Submitted*

Kobow K, Jeske I, Hildebrandt M, Buslei R, Hauke J, Hahnen E, **Siebzehnrübl FA**, Buchfelder M, Stefan H, Blücke I. Increased Reelin promoter methylation associates with granule cell dispersion in human temporal lobe epilepsy. *Submitted*

Weiss S, **Siebzehnrübl FA**, Kreutzer J, Blümcke I, Buslei R. nestin+/SMA+, sox2+ and musashi 1+ cells in the human adult pituitary: Evidence for a pituitary stem/progenitor cell? *To be submitted*

Coras R, Hösken A, Seufert S, Hauke J, Eyüpoglu IY, Reichel M, Tränkle C, **Siebzehnrübl FA**, Buslei R, Blümcke I, Hahnen E. Targeting TGF-ß mediated glioma cell migration and brain invasion by the PPAR-gamma agonist troglitazone (2007) Mol Cancer Ther. 6(6):1745-54

Buslei R, Holsken A, Hofmann B, Kreutzer J, **Siebzehnrübl F**, Hans V, Oppel F, Buchfelder M, Fahlbusch R, Blumcke I. Nuclear beta-catenin accumulation associates with epithelial morphogenesis in craniopharyngiomas (2007) Acta Neuropathol 113(5):585-90

(*) **Siebzehnrübl FA**, Buslei R, Eyüpoglu IY, Seufert S, Hahnen E, Blümcke I. Histone Deacetylase Inhibitors Increase Neuronal Differentiation in Adult Forebrain Stem and Progenitor Cells (2007) Exp Brain Res 176(4):672-8

Hölsken A, Eyüpoglu IY, Lüders M, Tränkle C, Dieckmann D, Hahnen E, Blümcke I, **Siebzehnrübl FA**. Experimental *ex vivo* therapy of malignant melanomas seeded into the brain using inhibitors of histone deacetylases (2006) Acta Neuropathol 112(2):205-15

Hahnen E, Eyüpoglu IY, Brichta L, Haastert K, Traenkle C, **Siebzehnrübl FA**, Riessland M, Hölker I, Claus P, Romstöck J, Buslei R, Wirth B, Blümcke I. Survival of Motor Neuron Gene activation by Suberoylanilide Hydroxamic Acid: Epigenetic Therapy for Spinal Muscular Atrophy (2006) J Neurochem 98(1):193-202

Eyüpoglu IY, Hahnen E, Tränkle C, Savaskan NE, **Siebzehnrübl FA**, Buslei R, Lemke D, Wick W, Fahlbusch R, Blümcke I. Experimental therapy of malignant gliomas using the inhibitor of class I histone deacetylases MS-275 (2006) Mol Cancer Ther 5(5):1248-1255

Buslei R, Kreutzer J, Hofmann B, Schmidt V, **Siebzehnrübl F**, Hahnen E, Eyüpoglu IY, Fahlbusch R, Blümcke I. Abundant hypermethylation of SOCS-1 in clinically silent pituitary adenomas (2006) Acta Neuropathol 111(3):264-271

Eyüpoglu IY, Hahnen E, Buslei R, **Siebzehnrübl FA**, Savaskan NE, Lüders M, Tränkle C, Wick W, Weller M, Fahlbusch R, Blümcke I. Suberoylanilide hydroxamic acid (SAHA) has potent anti-glioma properties in vitro, ex vivo and in vivo (2005) J Neurochem 93(4):992-999

Eyüpoglu IY, Hahnen E, Heckel A, **Siebzehnrübl FA**, Buslei R, Fahlbusch R, Blümcke I. Malignant gliomas induce neuronal cell death in an organotypic glioma invasion model (2005) J Neurosurg. (102):738-744

Buslei R, Nolde M, Hofmann B, Meissner S, Eyüpogly IY, **Siebzehnrübl F**, Hahnen E, Kreutzer J, Fahlbusch R. Common mutations of *β-catenin* in adamantinomatous craniopharyngiomas but not in other tumours originating from the sellar region (2005) Acta Neuropathol 109(6):589-597

Brichta L, Hofmann Y, Hahnen E, **Siebzehnrübl FA**, Raschke H, Blümcke I, Eyupoglu IY, Wirth B. Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy (2003) Hum Mol Genet 12(19):2481-2489

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