Regulation of adult neurogenesis in Huntington’s disease:
The role of TGF-beta1 signaling in the neurogenic niche
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Die Arbeit wurde angeleitet von: Prof. Dr. Inga Neumann und Prof. Dr. Ludwig Aigner
in der Klinik und Poliklinik für Neurologie der Universität Regensburg
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List of abbreviations

α-MEM  Alpha Modified Eagle Media
AD  Alzheimer’s disease
ALK  Activin like kinase
BDNF  Brain Derived Neurotrophic Factor
Bmi-1  B lymphoma Mo-MLV insertion region-1
BMP-2  Bone Morphogenetic Protein 2
BMP-4  Bone Morphogenetic Protein 4
bp  Base pair
BSA  Bovine Serum Albumine
BrdU  5-bromo-2-deoxyuridine
BT  Biotinylated
CAG  Cytosine, Adenine and Guanine
CBP  CREB-binding protein
CNTF  Ciliary neurotrophic factor
Cor  Cortex
CNS  Central Nervous System
CREB  cAMP response element-binding
CSF  Cerebrospinal fluid
ACSF  Artificial Cerebrospinal fluid
DAPI  4’,6-Diamidino-2-phenylindole
DG  Dentate Gyrus
DCX  Doublecortin
DNA  Deoxyribonucleic acid
DNI  Dystrophic Neuritic Inclusions
DMEM  Dulbecco’s Modified Eagle Media
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal Growth Factor
ES cell  Embryonic Stem cell
EPO  Erythropoietin
FBS  Fetal Bovine Serum
FGF  Fibroblast Growth Factor
FSGB  Fish Skin Gelatin Buffer
GABA  Gama Aminobutyric acid
GCL  Granule Cells Layer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth differentiation factors</td>
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<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GS-Domain</td>
<td>Glycin-Serine domain</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HC</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
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<tr>
<td>Hes</td>
<td>Hairy and enhancer of split</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIP1</td>
<td>Huntingtin Interacting Protein1</td>
</tr>
<tr>
<td>HAP1</td>
<td>Huntingtin-associated protein 1</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>HSCs</td>
<td>Hematopoietic Stem Cells</td>
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<tr>
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<td>Huntingtin Gene</td>
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<td>IT15</td>
<td>Interesting Transcript 15</td>
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<td>LAP</td>
<td>Latency Associated Protein</td>
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<tr>
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<td>Latent TGF-beta-binding proteins</td>
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<tr>
<td>Map2ab</td>
<td>Microtubule-associated Protein 2 Isoform a and b</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MH</td>
<td>Mad Homology</td>
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<td>NB</td>
<td>Neurobasal</td>
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<td>Nerve Growth Factor</td>
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<td>Neuronal Inclusion</td>
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<td>Neural Progenitor Cells</td>
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<tr>
<td>NSCs</td>
<td>Neural Stem Cells</td>
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<td>NeuN</td>
<td>Neuronal Nuclei</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory Bulb</td>
</tr>
<tr>
<td>Olig 1</td>
<td>Oligodendrocyte transcription factor1</td>
</tr>
<tr>
<td>Olig 2</td>
<td>Oligodendrocyte transcription factor2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>pCREB</td>
<td>Phospho cAMP response element-binding</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMFS</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
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<td>pSmad</td>
<td>phospho mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostra Migratory Stream</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SEL</td>
<td>Subependymal layer</td>
</tr>
<tr>
<td>SF</td>
<td>Scatter Factor</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
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<tr>
<td>SMI94</td>
<td>Antibody against Myelin Basic Protein</td>
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<tr>
<td>Sox2</td>
<td>SRY (sex determining region Y)-box 2</td>
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<tr>
<td>SSC</td>
<td>Saline Sodium Citrate</td>
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<td>mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA</td>
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<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
</tr>
<tr>
<td>Str</td>
<td>Striatum</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TgHD rats</td>
<td>Transgenic HD rats</td>
</tr>
<tr>
<td>TGF-beta1</td>
<td>Transforming growth factor beta1</td>
</tr>
<tr>
<td>TGF-beta2</td>
<td>Transforming growth factor beta2</td>
</tr>
<tr>
<td>TGF-beta3</td>
<td>Transforming growth factor beta3</td>
</tr>
<tr>
<td>TGF-bR1</td>
<td>Type 1 receptor Transforming growth factor beta</td>
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<tr>
<td>TGF-bR2</td>
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</tr>
<tr>
<td>TGF-bR3</td>
<td>Type 3 receptor Transforming growth factor beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
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1. Introduction

1.1. Adult neurogenesis

The renowned Spanish neuroanatomist Ramón y Cajal stated that “Once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres, the nerve paths are something fixed and immutable: everything may die, nothing may be regenerated”\(^1\). Therefore, it has been believed that no new neurons are generated in the adult brain and most of the common central nervous system (CNS) pathologies accompanied by neuronal loss cannot be restored. Amongst them are well known ones: Parkinson's disease accompanied by the degeneration of dopaminergic neurons in the substantia nigra, Alzheimer's disease with a neuronal loss in the cerebral cortex and certain subcortical regions and stroke where a certain brain area lacks oxygen supply followed by neuronal death. According to the above dogma, the vast majority of neurons in the mammalian brain are generated during embryonic development\(^2,3\). This statement stands true for most of the regions of the adult brain. However, this doctrine ended in 1965 when newly generated neurons were found in two specific regions of the adult brain: the subgranular zone (SGZ) in the dentate gyrus (DG) generates new granular neurons in granule cell layer (GCL) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle wall that gives rise to new cells that migrate along the rostral migratory stream (RMS) to become neurons in the olfactory bulb\(^4,5\) (Fig.1.1).
**Fig.1.1. Neurogenic niches in the adult human and in the rat brain.**
The dentate gyrus of the hippocampus and the subventricular zone are known to produce new neurons in the adult brain. From the subventricular zone of the lateral ventricle cells migrate via the rostral migratory stream to the olfactory bulb, where they differentiate into mature neurons and integrate. (Figure extracted from www.pubs.niaaa.nih.gov/publications/arh27-2/IMAGES/Page198.gif)

### 1.2. History of adult neurogenesis

A study suggested in 1912 by a Canadian scientist Ezra Allen, is considered for the preliminary document of mitotic activity in cells of the adult rodent central nervous system. Although there were some occasional reports on mitotic cells in the brain of adult mammals there were no convincing methods to prove that these new cells would differentiate into neurons and be functionally integrated. Joseph Altman and Gopal Das proposed the concept of persistent neurogenesis in the adult brain in 1965 where they used tritiated (H³) thymidine and autoradiography to suggest the production of new neurons in the hippocampus. Later on, it was demonstrated through autoradiography and electron microscopy that the newborn neurons in the hippocampus were structurally integrated. Many advanced techniques emerged in the 1990s. Instead of H³ thymidine, bromodeoxyuridine (BrdU) and retroviral labelling were used to monitor the newly dividing cells.
BrdU can be easily detected with immunohistochemical methods and visualized with bright-field and fluorescence microscopes\textsuperscript{16, 17}. Moreover, combination of the BrdU labelling method with specific antibodies against neuronal or glial cell markers has allowed the discrimination of newly generated neurons from glial cell type\textsuperscript{10, 18, 19}. With the help of these strategies, adult neurogenesis has been demonstrated in numerous mammalian species including humans\textsuperscript{20, 21}. Finally, the functional integration of the newly generated neurons into the existing neural network was confirmed demonstrating that these cells participate in long term potentiation (LTP), by illustrating synapse formation and expression of immediate early genes after stimulation of the hippocampal circuit\textsuperscript{16, 22, 23}.

1.3. Stem cells in the adult brain

In fully matured organisms most of the organs depend on the small local population of cells named as adult stem cells or somatic stem cells for their maintenance and their regenerative potential. Generally, these stem cell populations are established in a defined niche or microenvironment where they remain quiescent, proliferate slowly and produce another population of transient amplifying precursor cells. These transient amplifying cells are proliferating fast and migrate towards the ultimate destination where they undergo differentiation into an appropriate functionally mature cell type. Therefore, the potential of adult stem cells has to be tightly regulated for tissue homeostasis. In the adult brain new neurons are generated from neural stem cells and progenitor cells. These neural stem cells (NSCs) are multipotent in nature and have the capacity to self renew and give rise to neurons, astrocytes and oligodendrocytes\textsuperscript{24} (Fig 1.2). These adult stem cells can be isolated, expanded in culture and also be transplanted\textsuperscript{25}. 
Neural stem cells (NSC) are multipotent and have the capacity to self renew and give rise to lineage-restricted precursor cells, which further differentiate into the three major cell types of the CNS: neurons, astrocytes and oligodendrocytes.

1.4. Hippocampal neurogenesis

The hippocampus is a bilateral structure that plays a major role in processing and storage of new information. In the hippocampus, stem cells are located along the border between the granular cell layer (GCL) and the hilus known as subgranular zone (SGZ), where they produce cluster-forming precursor cells. From there, neuroblasts migrate into the GCL and become fully matured functional neurons, where they extend dendrites into the molecular layer (ML) and launch mossy fibers to the CA3 region$^{26, 27}$(Fig. 1.3). Following the principle ‘do or die’, the survival depends on how sufficiently the new cells are integrated into the neural circuit$^{28-30}$. From the neural stem cell to the mature neuron the cells go through defined steps of division, differentiation, migration and maturation. Using specific markers it is possible to investigate the stage specific changes of SGZ neurogenesis in detail$^{10, 31}$. Further, stem and progenitor cells from adult hippocampus produced neurons that generated action potentials, received functional GABAergic and glutamatergic synaptic inputs$^{32, 33}$.
Fig. 1.3. Processes of neurogenesis in hippocampal DG
During stage specific event of hippocampal neurogenesis, neural stem cells give rise to mature granular neurons through the production and development of intermediate precursors cells. (Modified from Encinas JM et al., 2006)

1.5. Neurogenesis in the SVZ-RMS-OB system

The newborn neurons generated in the OB originate from the subventricular zone (SVZ) of the lateral ventricle (LV). In the adult brain, newly generated SVZ young neurons migrate along the rostral migratory stream (RMS) and proceed to the olfactory bulb (OB). These neuronal cells integrate upon their arrival into the olfactory bulb as specific subtypes of interneurons. These subtypes are GABAergic granule cells, which represent the majority of the new OB neurons, and a very small number of dopaminergic periglomerular interneurons. The olfactory granule cells are inhibitory interneurons that make their dendritic connections to the mitral cells and to the middle tufted cells. The periglomerular neurons project their dendrites into the corresponding glomerulum and connect to the incoming olfactory...
axons from the sensory epithelium. It has been shown that these newly formed neurons are functionally integrated into the synaptic circuitry of the OB\textsuperscript{36, 38, 39} (Fig 1.4).

**Fig.1.4. Neurogenesis in the SVZ-RMS-OB system**
The DCX promoter EGFP transgenic mouse brain represents neurogenesis in the adult olfactory bulb (OB) that is initiated by the persistence of stem cells and progenitors in the subventricular zone (SVZ) of the lateral ventricle. The newly generated cells migrate through the rostral migratory stream (RMS) and into the olfactory bulb, where they differentiate into mature neurons (http://www.uni-regensburg.de/Fakultaeten/Medizin/Neurologie/forschung/zell.html).

At present, the functional significance of adult neurogenesis is not clear. However, many studies have shown that neurogenesis is involved in learning and memory. This has led to a hypothesis that suggests impairment in memory during aging and neurodegenerative diseases may involve abnormal neurogenesis. In the following chapters, these issues will be addressed in more detail.
Due to the general recognition and acceptance of adult neurogenesis, there has been an immense response from the scientific community, resulting in a large number of studies investigating how neurogenesis is regulated. Adult neurogenesis is a complex multistep process. This process includes proliferation, cell cycle exit, fate determination of adult neural progenitors and their differentiation, maturation and final integration into the neural circuits. Although, the precise mechanisms that generate new neurons in the adult brain remain elusive, a range of environmental-, behavioral-, genetic-, neuroendocrine-, neurochemical- and growth factors as well as cytokines have been shown to be involved in the regulation of adult neurogenesis. 

A number of stimuli have been shown to influence neurogenesis: in an enriched environment the animals are kept in housing conditions that are more similar to their natural environment. Such an enriched condition has given rise to increased neurogenesis and seems to play a neuroprotective role for newly generated neurons. Similar to enriched environmental conditions, wheel running- physical exercise has also been shown to boost hippocampal neurogenesis drastically through an increasing rate of progenitor proliferation. The animals that were exposed to an enriched environment and physical exercise showed improved motor skills and better performance in learning tasks. Stroke, a pathological situation in which blood supply to the brain is suddenly disrupted. It has been shown that stroke also stimulates the generation of new neurons. Epileptic seizure is another pathological situation, which arises from the abnormal excitation of neuronal networks in the brain. This epileptic pathological process has also been shown to provoke neurogenesis in the adult brain. Fluoxetine is an antidepressant drug that has recently been shown to improve neurogenesis during young adulthood.
Besides, it has been shown that neurogenesis in the hippocampus decreases with age, although in very old animals, there still remains a low-level of neurogenesis\textsuperscript{19, 46}. Stress is a physiological response to any kind of unpleasant events that provoke the hypothalamic pituitary axis (HPA) and raise the release and circulation of adrenal steroid hormones. Adrenal steroids may be one of the most important neurochemical regulators of neurogenesis. An increased plasma level of corticosterone, as it appears as a reaction to applied stress, has negative effects on hippocampal neurogenesis\textsuperscript{47-49}. However, this stress induced inhibition of precursor cell proliferation has been shown to be prevented by systemic administration of prolactin (PRL)\textsuperscript{50}.

1.7. Regulation of adult neurogenesis by signaling molecules

In mammalian tissue, typical homeostasis requires elaborately balanced interactions between cells and the network of secreted proteins. These reciprocal communications involve various extracellular cytokines acting via specific cell surface receptors. When the balance between the cells and the extracellular communication is dysregulated, pathogenesis can result \textsuperscript{51}. Growth factors are capable of controlling cellular growth, differentiation, maturation and survival. Numerous studies have been carried out to demonstrate that progenitors in the adult brain respond to growth factors. Intracerebroventricular infusion of epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) increased proliferation in the SVZ of the adult rats brain \textsuperscript{31}. Also insulin-like growth factor-1 (IGF-1) seems to be involved in the regulation of adult neurogenesis. Plasma levels of IGF-1 are increased by exercise and this promotes major increases in GCL precursor proliferation\textsuperscript{52}. Moreover, other studies have demonstrated that intracerebral infusion
of IGF increases both cell proliferation and neurogenesis in hypophysectomized rats. Like IGF-1, vascular endothelial growth factor (VEGF) also has a stimulatory effect on neurogenesis. Furthermore, a recent report demonstrated that granulocyte colony-stimulating factor (G-CSF) promotes proliferation of neural progenitors.

In contrast, members of the family of transforming growth factor beta (TGF-beta) are known to inhibit neurogenesis by blocking the proliferation of precursor cells in the adult brain. Therefore, TGF-betas and their downstream signaling are at the focus of attention to elucidate their involvement in adult neurogenesis. Bone morphogenic proteins (BMP) are extracellular signaling molecules that play diverging roles in neuronal development. Generally, the BMP molecules are characterized by their antagonistic action on neurogenesis. Noggin, e.g., is a soluble inhibitor for the BMP4 signal that promotes neurogenesis by blocking the BMP4 influence on stem cell proliferation.

1.8. Transforming growth factors

The TGF gene family expresses a set of structurally and functionally related polypeptides that include activins, bone morphogenic proteins (BMPs), the growth differentiation factors (GDFs), and a small group of pleotropic cytokines, the TGF-beta molecules. The TGF-beta name was coined in the year 1981 because of its transforming effect on rat kidney and fibroblast cell lines. TGF-betas have been implicated in cell proliferation, differentiation, migration, survival, apoptosis, extracellular matrix (ECM) formation, angiogenesis, metastasis, tumorogenesis, inflammation and tissue repair. There are three highly homologous isoforms of TGF-beta molecules: TGF-beta1, TGF-beta2 and TGF-beta3. Each of these three
isoform genes encodes an inactive precursor protein. Sequential processing to gives rise to the active TGF beta ligand. From the 391-amino-acid precursor form of TGF-beta1, the C-terminal 112 amino acids comprise the mature protein. The N-terminal peptide is the pro-domain, called the latency associated peptide (LAP). TGF-beta is secreted as a large latent complex composed of the active TGF-beta form covalently bound to LAP, which in turn is bound to a latent TGF-beta-binding protein (LTBP). Since the LTBP is linked to the extracellular matrix (ECM), the entire complex is stored in the extracellular space and provides a source of readily available ligand. Extracellular serine proteases cleave the LTBP and release the active ligand from LAP. The biologically active form of TGF-beta consists of a homodimer built out of two peptides each in size of 12.5 kD, which are linked through disulfide bonds.

1.8.1. The TGF-beta signaling pathway

The TGF-beta family members bind to their cognate heteromeric receptor complex, which consists of two types of transmembrane serine/threonine kinases known as type I (TGF-betaRI or ALK) and type II receptors (TGF-betaRII). These transmembrane receptors represent two families of serine/threonine kinase receptors of 53 to 65 kD and 80 to 95 kD, respectively. In mammals five isoforms of TGF-betaRI and seven isoforms of TGF-betaRII were identified. TGF-betaRIII (betaglycan and endoglin) is an indirectly signaling mediator which promotes the affinity of TGF-betaRII for TGF-beta2. In contrast, TGF-beta1 and TGF-beta3 bind directly to TGF-betaRII, a constitutively active kinase that leads to dimerization with the type I receptor and phosphorylation of the glycine-serine (GS) domain. Phosphorylation of the GS domain activates the C-terminal kinase domain, which phosphorylates and thereby activates receptor Smads (homologous proteins to the
Sma and Mad proteins from Caenorhabditis elegans and Drosophila melanogaster (R-Smads). Characteristically, all Smad proteins possess two domains, the MH1 and MH2 (mad homology) domains, the MH1 domain is located on the amino-terminus and the MH2 domain is located on the carboxy-terminus. Functionally, the MH1 is involved in protein-DNA interaction whereas the MH2 being responsible for the protein-protein interaction. Accordingly TGF-beta activates the phosphorylation of Smad2 and Smad3 while BMPs activates the phosphorylation of Smad1, Smad5 and Smad8. The phosphorylated R-Smads dimerize with Co-Smad (Smad4) and transloctate to the cell nucleus where they exert their function as transcription factors. TGF-beta1 stimulation leads to the nuclear translocation of the phosphorylated Smad 2/3 and of the Co-Smad 4 complex that activates the inhibitory I-Smads (Smad6 and Smad7). These activated I–Smads act as an antagonist for TGF-betaRII mediated downstream signal by blocking the receptor accessibility to R-Smads (Fig 1.5)\(^{57, 58, 67, 68}\).

**Fig.1.5. Receptor mediated TGF-beta1 signaling**

TGF-beta ligand binds to TGF-betaRII that complexes with and activates TGF-betaRI. This induces the downstream Smad-mediated signal transduction (Aigner and Bogdahn, 2008).
1.8.2. TGF-beta expression in the normal and pathological brain

TGF-betas are involved in various physiological and pathological processes in the CNS. All three isoforms of TGF-beta are expressed within the nervous system, in neurons and in glial cells\(^58, 69, 70\). Most of the current knowledge about the expression of TGF-beta in the CNS comes from studies of the development. In the adult, TGF-beta2 and TGF-beta3 can be found in all areas of the CNS\(^58\). TGF-beta1 is widely expressed in the choroid plexus and in the meninges and its expression is drastically up regulated, in the CNS during injury and neurodegeneration\(^71-74\) where it is secreted predominantly by activated microglial cells\(^75\). In addition, cultivated neurons and astrocytes have been shown to secret TGF-beta1\(^76\). In brain pathology, TGF-beta1 is involved in coordinating the inflammatory responses and brain recovery. TGF-beta1 and TGF-beta2 are also involved in brain-tumor development and progression, in particular of high-grade gliomas\(^58, 77-81\).

1.8.3. TGF-beta signal transduction in the brain

TGF-beta receptors are expressed in the brain\(^69, 82\). Expression of TGF-betaRII mRNA is clearly reported in most of the brain areas such as cerebral cortex, midbrain, cerebellum, and brainstem\(^58, 69\). Concerning the cell type specificity it is expressed in neurons, astroglia, microglia, endothelial cells, and other non-neuronal cells found in the choroid plexus\(^58, 83, 84\). Surprisingly, in a recent in vivo imaging study from the group of Wyss-Coray on a Smad-responsive luciferase transgenic mouse, a high level of downstream signaling activity of TGF-beta was noticed in the intact adult brain\(^85\). Subsequently, it was demonstrated that the expression of TGF-betaRI and II mRNA and of the TGF-betaRII protein in neural stem and progenitor cells isolated from the SVZ of the adult brain\(^82\).
1.8.4. Role of TGF-beta in the brain

TGF-beta displays diverse functions in the adult brain. TGF-beta1 is a global immune modulator and it plays a crucial role in neuroprotection. TGF-beta1 has been shown to promote the survival of neurons\textsuperscript{86-88}. In addition to its other effects and probably depending upon its discrete cellular environment, TGF-beta1 inhibits proliferation of astrocytes but induces their differentiation\textsuperscript{89}. TGF-beta1 has been shown also to exert a negative effect on the proliferation of microglia\textsuperscript{90}.

1.8.5. Regulation of adult neurogenesis by TGF-beta1

Recently, the regulation of adult neurogenesis by experimentally induced level of TGF-beta1 was demonstrated. Thus infusion of TGF-beta1 into the ventricles of adult rat brain revealed a reduced amount of proliferating cells in the hippocampus and in the SVZ. Further, infusion of TGF-beta1 lowered the number of DCX expressing neuronal precursor in these neurogenic niches. Besides, in an in vitro study treatment of TGF-beta1 in the neurosphere cultures reduced the proliferation of stem cell and progenitor cells and induced a shift to G0 phase of the cell cycle\textsuperscript{82}. Subsequently, a study from the Wyss Coray group has confirmed these findings in the brains of transgenic animals, that over-express TGF-beta1 under the control of the glial fibrillary acidic protein promoter in astrocytes\textsuperscript{91}. In addition, other reports mainly focused on the intermediate and late stages of the neurogenic processes and described that TGF-beta1 elevates neuronal differentiation and survival. For example, injection of adenoviral vectors expressing TGF-beta1 into the SVZ of the adult rats brain increased the expansion of DCX-expressing newly born immature neurons\textsuperscript{92}. Intranasal administration of TGF-beta1 in adult mice after stroke promoted the number of newborn DCX and NeuN positive neurons via reducing the
cell death process\textsuperscript{93}. There is an increased number of newly born PSA-NCAM positive neurons in the hippocampus of adrenalectomized rats brain where it has been correlated with an induced secretion of TGF-beta 1 by activated microglia\textsuperscript{94}. Moreover, TGF-beta1 is an important neuroprotective factor that can prevent neuronal damage from almost all kind of brain damage including neurodegenerative disease\textsuperscript{72, 86, 95, 96}.

1.8.6. Enhanced TGF-beta1 levels and impaired neurogenesis in neurodegenerative disorders

Neurodegenerative disorders are devastating hereditary and sporadic conditions which are characterized by progressive loss of neuron structure and function, ultimately leading to the death of selective neuronal populations in specific brain areas. Many neurodegenerative disorders including Alzheimer’s disease (AD), Parkinson’s disease (PD) and Huntington’s disease (HD) occur as a result of degeneration of neurons due to the toxicity of protein aggregation. So far no promising treatments are available to eradicate these disease conditions. During past decades series of reports have demonstrated impaired neurogenesis in the brain under degenerative conditions occurring with diseases such as AD, PD and HD\textsuperscript{97-99}. Therefore, understanding the regulation of neurogenesis in degenerative brains is of crucial importance for therapeutic intervention. In most of the neuropathological conditions, it has been shown that specifically the inflammatory cytokines and their downstream signaling are altered\textsuperscript{83}. For example, while neurogenesis is impaired in the diseased brain of patients with AD and HD, the pleiotropic cytokine TGF-beta1 and their downstream signaling components are
elevated\textsuperscript{71, 96}. This alteration in cytokine expression and its subsequent signaling cascades might be playing a crucial role in impaired neurogenesis. Therefore, in the following chapters some key issues of the pathologies of Huntington’s disease will be described.

1.9. Huntington’s disease

Huntington’s disease, also termed Huntington chorea is an inherited autosomal dominant disorder resulting from an expansion of the CAG repeats within the Huntington gene (Htt) located on chromosome 4\textsuperscript{100}. The expansion of the CAG repeats causes polyglutamine stretches on the huntintin protein that induces progressive degeneration of neurons primarily in the striatum and in the cortex\textsuperscript{101}. This neuronal loss leads to cognitive deficits, impaired motor functions and psychiatric disturbances\textsuperscript{102, 103}. Thus far there are no treatments available to alleviate this devastating disease.

1.9.1. Clinical aspects of Huntington’s disease

Huntington’s disease is a progressive neurodegenerative disorder characterized by chorea, involuntary body movements and dementia. Psychiatric symptoms like depression are known to occur during the onset of disease\textsuperscript{104}. In general, the mood changes, irritability, anger and depression are commonly observed with minimal motor skills. As HD progresses, the physical, intellectual and emotional symptoms become more severe. In most of the cases, characteristic choreiform movements of the head, neck, arms and legs are more visible. In juvenile forms of HD, the patient will often suffer from muscle rigidity and bradykinesia without showing symptoms for chorea\textsuperscript{105}. These conditions are known as ‘Westphal
variant’ of HD and affect 6% of HD patients\textsuperscript{106}. Weight loss is one of the features of progressed form of HD due to poor food intake.\textsuperscript{107, 108} As a result of vulnerable dementia and progressive motor dysfunction, patients with advanced HD may become unable to walk, have poor dietary intake\textsuperscript{108}, eventually arrest in locomotion and become unable to care for themselves, eventually requiring long-term intensive care. Final and fatal complications may result from pathology related to serious falls, poor nutrition, infection, choking, inflammation of the lungs and heart failure (http://hdlighthouse.org).

1.9.2. History of Huntington’s disease

The word chorea in Greek means dance. It refers to the classes of movement disorders caused by destruction of the basal ganglia and describes the associated uncontrolled body movements. The first observation of chorea in childhood was reported by a British physician, Thomas Sydenham (1624-1689), but it was distinct from Huntington’s disease and associated with rheumatic fever also know as Sydenham chorea and as St. Vitus’ dance. Later on, Huntington’s disease or Huntington’s chorea was precisely described as an uncontrolled movement disorder in association with dementia in 1872 by George Summer Huntington, an American physician (Fig 1.6). While chorea movement disorders had been well recognized, Huntington’s disease expels unique features like the hereditary in nature, mutation in Htt, late onset and neuronal loss in the striatum and the cortex. Huntington’s disease is primarily an adult disorder, with an average onset between 35 and 42 years\textsuperscript{109}. However, the disease can encounter at any age and 6% are juvenile forms\textsuperscript{110}. The age of onset is closely correlated with the CAG repeat length of the mutant form of the htt gene\textsuperscript{111}.
1.9.3. Epidemiology of Huntington’s disease

Huntington’s disease is the most prevalent disorder in a family of several neurodegenerative diseases that are caused by a poly-glutamine expansion in the huntingtin protein. HD affects male and female with the same frequency\textsuperscript{112}. The highest prevalence is in Europe and North America, with 4-8 cases per 100000 people\textsuperscript{113}. Venezuela has the highest HD population in the world\textsuperscript{114}. This emerged to build the foundation of the Venezuela Huntington’s disease organization, which ultimately led to the identification of a transcript called IT15 (interesting transcript 15) located on the 4th chromosome which was shown to be associated with Huntington’s disease\textsuperscript{115}. Ten years later, in 1993, the gene was isolated and characterized and then called the HD gene or huntingtin (htt) by The Huntington’s Disease Collaborative Research Group. Mutations in the htt gene give raise to the polyglutamine stretches in the N-terminal region of the huntingtin protein\textsuperscript{100}.

1.9.4. Localization and function of the physiological Huntingtin protein

The human huntington gene contains 67 exons spanning around 200 kb in size and is located in the p arm of chromosome 4 at position 4p16.3 (Fig 1.7). This
gene contains a particular DNA segment known as a CAG trinucleotide repeat. This segment is made up of a series of three DNA building blocks (Cytosine, Adenine and Guanine) that appear multiple times in a row\textsuperscript{100}. Normal individuals have 10 to 35 CAG segment repeats. People with 36 to 40 CAG repeats may or may not develop the signs and symptoms of Huntington's disease while people with more than 40 repeats have an almost 100\% possibility to develop the disorder\textsuperscript{112,116}. Htt has very rare homology to other proteins and its functions are poorly understood.

Fig.1.7. Location of the huntingtin gene
Human chromosome 4 with the location of the HD gene indicated at the top of the short arm. (http://ghr.nlm.nih.gov/dynamicImages/chromomap/htt.jpeg)

Htt is widely expressed within the body with the highest levels in the brain and the testis. Within the brain regions, it can be found in highest levels in the cerebellar cortex, the striatum and the hippocampus\textsuperscript{117,118}. While the direct function of htt is not yet known, it is apparently required for normal embryogenesis, since htt knockout animals die at an early developmental stage\textsuperscript{119}. Conditional knockout studies have demonstrated that htt plays an essential role during postnatal development, as the inactivation of the gene in the brain and in the testis leads to degeneration of these two organs\textsuperscript{120}. Furthermore, htt is required for neuronal survival\textsuperscript{120-122}. There are
significant numbers of protein partners that interact with the huntingtin protein. The mainly known htt interaction proteins are involved in transcription and cellular trafficking. At present, around 100 interacting proteins have been listed to interact with huntingtin protein. From the list, huntingtin-associated protein 1 (HAP1) and huntingtin Interacting Protein 1 (HIP1) are extensively characterized for their interaction with htt$^{123,124}$. Concerning the neuronal survival, normal huntingtin protein known to up-regulate transcription and traffic of BDNF through its interaction with HAP1$^{125}$ but the underlying molecular mechanisms that controls the expression of BDNF are not clear.

1.9.5. Expanded CAG repeats in the huntingtin gene

In the HD gene, the number of CAG repeats plays a critical role for its functions. More than 40 CAG repeats in the HD allele definitively lead to a misfolding, loss of function and toxic protein aggregation. The increase in length of CAG repeats correlates with fast onset and high grade of HD pathology$^{116}$. However, the mechanism of polyglutamine expansion and its pathogenic role are unclear. In neurons, it has been proposed that missfolded htt aggregates translocate to nuclei and form neuronal inclusions (NI) and induce caspase mediated apoptotic cell death pathways$^{126,127}$. Recent studies have shown that altered huntingtin can trap some proteins and dislocate them from their original locations thus preventing them from functioning within nerve cells$^{128}$. Thus a study showed that Htt interferes with the function of CREB-binding protein$^{129}$.
1.9.6. Neuropathological hallmarks of HD

The loss of neurons in the HD brain starts in the striatal region. The striatum is the part of basal ganglia that contains medium spiny projecting neurons (MSN)\(^{130}\). In the HD brain, these neurons are most severely affected, resulting in atrophy of the striatum, first in the caudate nucleus, then in the putamen. The second hot spot of neurodegeration in HD is the cortex. Neurons in layer V and VI of the cortex projecting to the striatum are mostly affected. Characteristic polyQ protein aggregates are accounted for dysfunctions in the HD brain\(^ {131}\). These aggregates, which are ubiquitinated, are called neuronal nuclear inclusions (NII) or dystrophic neuritic inclusions (DNIs), depending on their sub-cellular localization. Inclusions are mainly found in the striatum and in the cortex\(^ {132}, 133\). Their direct effect on the neurodegeneration is still under debate, as both defensive and toxic functions have been described in HD but also in other neurodegenerative diseases such as the Lewy bodies in Parkinson’s disease and the amyloidal plaques in Alzheimer’s disease.

Pathological hallmarks of HD include marked decreases in volume in both the caudate nucleus and putamen, leading to the significant increase in lateral ventricle volume\(^ {134}, 135\). There are some studies using MRI techniques in HD patients that have also indicated extrastriatal differences, for example hippocampal atrophy\(^ {136}\). Hippocampal atrophy and hippocampal dysfunction are the most common abnormalities in cognitive deficits. Significant hippocampal volume loss has been found in most of the patients with comorbid depression\(^ {137}\). However in HD pathology, the effects of hippocampal volume loss on comorbidity and cognitive decline have not yet been studied in detail.
1.9.7. Experimental models of Huntington's disease

1.9.7.1. Acute models for Huntington's disease

Injection of amino acids such as N-methyl- D-aspartate (NMDA), quinolinic acid, and 3-nitro propionic acid into animals leads to neuronal loss in different regions of the brain including the striatum that is believed to be relevant with HD pathology\textsuperscript{138-140}. These chemicals are known to accelerate glutamate receptor activation and cause mitochondrial dysfunction in neurons of the striatum. These chemically induced models are still useful tools to test therapeutic strategies which can delineate the acute neuronal loss in the striatum. However these models do not mirror the progressive chronic neuronal degeneration and molecular changes along the disease progression.

1.9.7.2. Transgenic models of Huntington's disease

For HD, several transgenic models have been developed in different organisms ranging from nematodes to primates. The nematode Caenorhabditis elegans is the simplest genetic animal model of PolyQ neurotoxicity, in which the N-terminal 171 amino acid fragment of human huntingtin protein containing an expanded polyglutamine tract (150Qs) is expressed in neurons and this model displays neurodegeneration\textsuperscript{141}. PolyQ-expressing fruit flies form nuclear inclusions and undergo a progressive neurodegeneration\textsuperscript{142,143}. The major breakthrough in the field of Huntington’s disease has from the development of transgenic lines that express the exon1 of htt with 115 CAG (R6/1) or 155 CAG (R6/2) repeats and develop progressive behavioural symptoms and neuropathology\textsuperscript{144}. These animals display an early onset of HD pathology, have a shorter life span and die within the first two to four months\textsuperscript{144}. Recently, a transgenic rat model of Huntington's
disease (tg HD rat) was developed by von Hörsten and colleagues. This model carries a truncated huntingtin cDNA fragment encoding for 51 CAG repeats under the control of the rat huntingtin promoter. TgHD rats exhibit an adult-onset of the neurological HD phenotype characterized by elevated anxiety, cognitive impairment and slowly progressive motor dysfunction. Furthermore, these behavioral manifestations are accompanied by typical histo-pathological alterations such as NII in the basal ganglia and in the hippocampus. Besides, tgHD rats suffer from brain mitochondrial dysfunction and degeneration of medium spiny neurons. In addition to their progressive behavioural impairments and neuropathological signs, tgHD rats have an increased lethality starting at about 15 months of age. The slowly evolving pathology thus reflects more faithfully the human HD condition and permits a detailed analysis of progressive alterations in the brain organization and function. Moreover, it provides a window of opportunity to scrutinize the impact of endogenous or induced cellular plasticity and/or restorative processes during various phases in the course of the pathology.

1.9.8. Neurogenesis in Huntington’s disease

In the context of Huntington’s disease, it has been shown that neurogenesis is impaired in the hippocampus of transgenic R6 mouse lines of Huntington’s disease but it is increased in the SVZ of chemically induced acute models of the disease and HD patients. However, the impairment of adult neurogenesis at different stages of the disease progression in the HD brain has been not characterized. Hence, this study aims to characterize the regulation of hippocampal neurogenesis in different clinical phases of HD by using a transgenic HD rat model which resembles the human HD pathology.
2. Aim of the study

In the mammalian brain, new neurons are continuously generated in the hippocampus and in the SVZ throughout the adulthood. This process includes neural stem and progenitor cells maintenance, proliferation, neuronal differentiation, neuronal integration and survival. The exact molecular cues governing neurogenesis in these distinctive neurogenic niches are not known. The main objective of this thesis is to address the role of TGF-beta1 in regulation of adult neurogenesis in the normal brain and in the degenerative brain. This study was focused on the characterization of the endogenous level of receptor mediated TGF-beta1 signaling in the stem cell niche of the healthy brain. The stem and progenitors cells in the neurogenic niche are elaborately analyzed for the TGF-beta1 signaling in association with their cell proliferative and differentiation potentials. Further, the normal adult stem cell niche was challenged with elevated levels of TGF-beta1 by using the intracerebroventricular infusion and transgenic animal model systems. Therefore, it allows measuring the influence of TGF-beta1 on the regulation of stem and progenitor cells in the context of neurogenesis per se.

Adult neurogenesis is inhibited in neurodegenerative disorder such as Huntington’s disease but the progression of cellular events and molecular mechanisms that influence neurogenesis in HD brain are poorly understood. Strikingly, the expression of TGF-beta1 and TGF-beta signaling components are elevated in the degenerating HD brain. Therefore, we hypothesize that TGF-beta1 might be involved in the stem cell niche remodelling in HD brains. Hence, the present study investigated the regulation of neurogenesis in transgenic HD models at different clinical phases and scrutinized a correlation between observed neurogenic modulations and alterations in TGF-beta signaling.
3. Methods

3.1 Materials

3.1.1 Expendable materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotic minipumps</td>
<td>ALZET, Durect Corp., Cupertino, USA</td>
</tr>
<tr>
<td>BD Discardit™ II syringes</td>
<td>Becton Dickinson, Heidelberg</td>
</tr>
<tr>
<td>BD Plastikpak™ syringes (1ml)</td>
<td>Becton Dickinson, Heidelberg</td>
</tr>
<tr>
<td>Cell culture flasks (50ml, 250ml, 500ml)</td>
<td>TPP, Switzerland</td>
</tr>
<tr>
<td>Combitips</td>
<td>Greiner Bio-One GmbH, Frickenhausen</td>
</tr>
<tr>
<td>Cover glasses Menzel (Ø 13mm)</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td>Disposable gloves</td>
<td>VWR, Darmstadt</td>
</tr>
<tr>
<td>Disposable gloves</td>
<td>Hartman, Heidenheim</td>
</tr>
<tr>
<td>Disposable gloves</td>
<td>Semperit Austria</td>
</tr>
<tr>
<td>Cryo Tubes</td>
<td>Nunc, Denmark</td>
</tr>
<tr>
<td>Hyperfilm</td>
<td>AmershamPharmacia, Freiburg, Germany</td>
</tr>
<tr>
<td>Microscope slides</td>
<td>Menzel GmbH &amp; Co KG, Braunschweig</td>
</tr>
<tr>
<td>Nitrocellulose membrane</td>
<td>Schleicher and Schuell, Dassel, Germany.</td>
</tr>
<tr>
<td>Pipette tips</td>
<td>Sarstedt, Nürmbrecht</td>
</tr>
<tr>
<td>Pipette tips with filter</td>
<td>Biozym, Hessisch Oldendorf</td>
</tr>
<tr>
<td>Syringe-Filter (0.22µm)</td>
<td>TPP, Switzerland</td>
</tr>
<tr>
<td>Scalpels</td>
<td>Omnibus, Schubert &amp; Weiß, München</td>
</tr>
<tr>
<td>Test plates (6-well, 24-well, 96-well)</td>
<td>TPP, Switzerland</td>
</tr>
<tr>
<td>Tubes</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td></td>
<td>Falcon BD, Heidelberg</td>
</tr>
<tr>
<td></td>
<td>Gibco BRL, Karlsruhe</td>
</tr>
<tr>
<td></td>
<td>Sarstedt, Nümbrecht</td>
</tr>
</tbody>
</table>
3.1.2 Chemicals for in vivo immunological procedure

Acepromazine  
Vectastain Elite ABC kit  
BrdU  
BSA  
3,3’-Diaminobenzidine (DAB)  
Ethylene glycol  
Formamide  
Glycerol  
HCl  
H₂O₂  
Ketamine  
Methanol  
NaCl  
NaOH  
NaH₂PO₄  
Na₂HPO₄  
Nuclear  
Neo-Mount  
NiCl₂  
Paraformaldehyde (PFA)  
Prolong Antifade kit  
Teleostean gelatin  
Topro3  
Triton X 100  
Sucrose  
Tissue Tek  
Tris-Base  
xylazine

WDT, Garbsen, Germany  
Vector Laboratories, Burlingame, USA  
Sigma-Aldrich, Taufkirchen  
Sigma-Aldrich, Taufkirchen  
Vector Laboratories, Burlingame, USA  
AppliChem, Darmstadt  
Merck, Darmstadt  
Merck, Darmstadt  
Vector Laboratories, Burlingame, USA  
Merck, Darmstadt  
WDT, Garbsen, Germany  
Merck, Darmstadt  
Sigma-Aldrich, Taufkirchen  
Sigma-Aldrich, Taufkirchen  
Merck, Darmstadt  
Merck, Darmstadt  
Merck, Darmstadt  
Merck, Darmstadt  
Vector Laboratories, Burlingame, USA  
Sigma-Aldrich, Taufkirchen  
Invitrogen Molecular Probes™ Eugene, Oregon, USA  
Sigma-Aldrich, Taufkirchen  
Molecular Probes, USA  
Sigma-Aldrich, Taufkirchen  
Sigma-Aldrich, Taufkirchen  
Sakura finetek, USA  
Sigma-Aldrich, Taufkirchen  
WDT, Garbsen, Germany
3.1.3 Chemicals for Western blot

- 30% Acryamide/Bisacryamide solution: Sigma-Aldrich, Taufkirchen
- Ammonium Persulfate (APS): Sigma-Aldrich, Taufkirchen
- Aprotinin: Sigma-Aldrich, Taufkirchen
- Bovine Serum Albumine (BSA): Sigma-Aldrich, Taufkirchen
- Bicinonic Acid (Lowry): Sigma-Aldrich, Taufkirchen
- Bromphenol Blue: Sigma-Aldrich, Taufkirchen
- Copper Sulfate: Sigma-Aldrich, Taufkirchen
- Dithiothreitol (DTT): Sigma-Aldrich, Taufkirchen
- ECL plus: Amersham Pharmacia, Freiburg, Germany
- EDTA: Merck, Darmstadt
- Ethanol: Merck-Schuchard, Hohenbrunn
- Glycin: Merck, Darmstadt
- Beta-Mercaptoethanol: Sigma-Aldrich, Taufkirchen
- SDS: Merck-Schuchard, Hohenbrunn
- Tetramethylethylenediamine (TEMED): Sigma-Aldrich, Taufkirchen
- Tween-20: Sigma-Aldrich, Taufkirchen
- NP40 (IGEPAL): Sigma-Aldrich, Taufkirchen
- Ponceau Solution: Sigma, Germany
- PMSF: Sigma-Aldrich, Taufkirchen
- Pepstatin: Sigma-Aldrich, Taufkirchen
- Protease inhibitors: Invitrogen, USA

3.1.4 Cell culture medias

**Proliferation media**

Neurobasal Medium (Gibco, Karlsruhe) containing:

- 100µg/ml Penicillin/Streptomycin: PAN Biotech GmbH, Aidenbach, Germany
- 200mM Glutamin: PAN Biotech GmbH, Aidenbach, Germany
- 1x B27: Gibco BRL, Karlsruhe, Germany
- 20ng/ml Fibroblast Growth Factor-2: R&D Systems, Wiesbaden-Nordenstadt, Germany
20ng/ml Epidermal Growth Factor  
R&D Systems, Wiesbaden-Nordenstadt, Germany
2µg/ml Heparin  
Sigma-Aldrich, Taufkirchen, Germany

**Differentiation media**

Neurobasal Medium Gibco containing:
- 1x B27  
  Gibco BRL, Karlsruhe, Germany
- 100µg/ml Penicillin/Streptomycin  
  PAN Biotech GmbH, Aidenbach, Germany
- 200mM Glutamin  
  PAN Biotech GmbH, Aidenbach, Germany
- 5% Fetal Calf Serum (FCS)  
  PAN Biotech GmbH, Aidenbach, Germany

**3.1.5. Other reagents for cell culture**

- Accutase  
  PAA, Pasching, Austria
- Bone Morphogenic Protein 2/4  
  R&D Systems GmbH, Wiesbaden-Nordenstadt
- Bromphenol Blue  
  Sigma-Aldrich, Taufkirchen
- B27 supplement  
  Gibco BRL, Germany
- Dispase II  
  Boehringer, Germany
- DMEM/F12  
  Gibco BRL, Germany
- Dnase I  
  Worthington Biochemicals, England
- Dulbecco’s phosphate buffered saline  
  Sigma, Germany
- Dulbecco’s PBS  
  Gibco, Karlsruhe
- Fetal calf serum (FCS)  
  PAN, Germany
- Glucose  
  Merck, Germany
- Hank’s Balanced Salt Solution  
  PAN, Germany
- Heparin  
  Sigma, Germany
- IMEM  
  Gibco BRL, Germany
- Laminin  
  Sigma, Germany
- L-glutamine  
  PAN, Germany
- Neurobasal Medium (NB)  
  Gibco BRL, Germany
- Papain  
  Worthington Biochemicals, England
- Penicillin/streptomycin  
  PAN, Germany
- Poly-L-ornithine  
  Sigma, Germany
- Trypan Blue  
  Sigma, Germany
### 3.1.6 Buffer, solutions and stock solutions

<table>
<thead>
<tr>
<th>Buffer/Solution Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tris Buffered Saline (TBS)</strong></td>
<td>800 ml dH2O 8g NaCl 3g Trisbase HCl adjust to pH 7.4</td>
</tr>
<tr>
<td><strong>Borate Buffer (0.1M)</strong></td>
<td>3.08g boric acid 450 ml H2O 5N NaOH to pH 8.5 Final volume to 500 ml</td>
</tr>
<tr>
<td><strong>PBS (0.1M)</strong></td>
<td>500ml 0.2M Phosphate Buffer 500ml dH2O 9g Natriumchloride</td>
</tr>
<tr>
<td><strong>Phosphate Buffer (0.2M)</strong></td>
<td>1L dH2O 5.52g NaH2PO4, water free 21.9g Na2HPO4, water free 10ml 10xPBS</td>
</tr>
<tr>
<td><strong>Cryo Protective solution</strong></td>
<td>250 ml Glycerin 250 ml Ethylenglycol 500 ml 0.1M PO4 buffer.</td>
</tr>
<tr>
<td><strong>Fish Skin Gelatin Buffer (FSGB)</strong></td>
<td>0.1M Tris-HCl, pH 7.5 0.15M NaCl 1% w/v BSA 0.2% v/v Fish Skin Gelatin</td>
</tr>
<tr>
<td>Solution/Buffer</td>
<td>Composition/Components</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>0.1% v/v Triton X-100</td>
<td></td>
</tr>
<tr>
<td>DAB solution</td>
<td>0.25 mg/ml 3, 3’-diaminobenzidine</td>
</tr>
<tr>
<td></td>
<td>0.01% (v/v) H2O2,</td>
</tr>
<tr>
<td></td>
<td>0.04% (w/v) NiCl2</td>
</tr>
<tr>
<td>Donkey serum blocking buffer</td>
<td>0.1 M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>0.15 M NaCl</td>
</tr>
<tr>
<td></td>
<td>3% donkey serum</td>
</tr>
<tr>
<td></td>
<td>0.1% Triton X-100</td>
</tr>
<tr>
<td>4% Paraformaldehyde (PFA)</td>
<td>250 ml dH2O</td>
</tr>
<tr>
<td>For perfusion</td>
<td>0.5 ml 10M NaOH</td>
</tr>
<tr>
<td></td>
<td>20 g PFA</td>
</tr>
<tr>
<td></td>
<td>250 ml 0.2M PO4 buffer</td>
</tr>
<tr>
<td>4% Paraformaldehyde (PFA)</td>
<td>4% PFA</td>
</tr>
<tr>
<td>For fixation of cells</td>
<td>2.5mM NaOH</td>
</tr>
<tr>
<td></td>
<td>0.4mM CaCl2</td>
</tr>
<tr>
<td></td>
<td>50mM Sucrose</td>
</tr>
<tr>
<td></td>
<td>0.1M NaH2PO4</td>
</tr>
<tr>
<td>Protein Isolation Buffer</td>
<td>0.7% IGEPAL</td>
</tr>
<tr>
<td>(Fei He et al. 2005 modified)</td>
<td>50mM Tris HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>0.1mm EDTA pH 8.0</td>
</tr>
<tr>
<td></td>
<td>250mM NaCl</td>
</tr>
<tr>
<td></td>
<td>10% Glycerol</td>
</tr>
<tr>
<td></td>
<td>0.2mm Na2VO4</td>
</tr>
<tr>
<td></td>
<td>50mM NaF</td>
</tr>
<tr>
<td></td>
<td>1mM PMSF</td>
</tr>
<tr>
<td></td>
<td>10mM DTT</td>
</tr>
<tr>
<td></td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>Sample buffer for proteins - WB</td>
<td>1M Tris-HCl, pH 6.8</td>
</tr>
<tr>
<td>Component</td>
<td>Composition</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Resolving gel buffer -WB</td>
<td>2M Tris-HCl pH 8.0</td>
</tr>
<tr>
<td></td>
<td>10% SDS</td>
</tr>
<tr>
<td></td>
<td>H₂O bidest</td>
</tr>
<tr>
<td>Stacking gel buffer -WB</td>
<td>1M Tris-Cl, pH 6.8</td>
</tr>
<tr>
<td></td>
<td>10% SDS</td>
</tr>
<tr>
<td></td>
<td>H₂O bidest</td>
</tr>
<tr>
<td>Electrophoresis buffer (1L)-WB</td>
<td>25mM Tris Base (3g)</td>
</tr>
<tr>
<td></td>
<td>14.4g Glycin</td>
</tr>
<tr>
<td></td>
<td>1% SDS</td>
</tr>
<tr>
<td>Blotting Buffer-WB</td>
<td>25mM Tris Base (3.03g/L)</td>
</tr>
<tr>
<td></td>
<td>150mM Glycine (11.26g/L)</td>
</tr>
<tr>
<td>PPD (100 ml)</td>
<td>0.01% Papain</td>
</tr>
<tr>
<td></td>
<td>0.1% Dispase II</td>
</tr>
<tr>
<td></td>
<td>0.01% Dnase I</td>
</tr>
<tr>
<td></td>
<td>149 mg MgSO₄·7H₂O in Hank’s Balanced Salt Solution w/o Ca²⁺/Mg²⁺</td>
</tr>
<tr>
<td>Running buffer for WB</td>
<td>3.02g Tris-Base</td>
</tr>
<tr>
<td></td>
<td>14.4g glycerin</td>
</tr>
<tr>
<td></td>
<td>1.0g SDS</td>
</tr>
<tr>
<td></td>
<td>dissolve in 0.8l H₂O, set at pH 8.3 with 2M HCl and fill up to 1l with H₂O</td>
</tr>
<tr>
<td>30% Succrose</td>
<td>400ml 0.1M PO₄</td>
</tr>
<tr>
<td></td>
<td>150g Sucrose</td>
</tr>
</tbody>
</table>
SSC  
3.0M NaCl  
0.3M NaCl x 2H2O  
QS 500ml H2O, pH 7.0

TBST  
TBS+0.25% TWEEN

### 3.1.6. Primary antibodies

<table>
<thead>
<tr>
<th>List of Antibodies</th>
<th>Marker</th>
<th>Dilution for Histology</th>
<th>Dilution for WB</th>
<th>Source</th>
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<tbody>
<tr>
<td>Rat anti-BrdU</td>
<td>Base analogue</td>
<td>1:500</td>
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<td>Oxford Biotechnology, UK.</td>
</tr>
<tr>
<td>Mouse anti-PCNA</td>
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<td>1:500</td>
<td></td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>Rabbit anti-GFAP</td>
<td>Astrocytes</td>
<td>1:100</td>
<td></td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>Guinea pig anti-GFAP</td>
<td>Astrocytes</td>
<td>1:500</td>
<td></td>
<td>Progene, Germany</td>
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<tr>
<td>Mouse anti rat-Nestin</td>
<td>Stem cell</td>
<td>1:500</td>
<td></td>
<td>Pharmingen, USA</td>
</tr>
<tr>
<td>Goat anti-Sox2</td>
<td>Stem cell</td>
<td>1:500</td>
<td></td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>IgM mouse anti- A₂B₅</td>
<td>Glial progenitor</td>
<td>1:200</td>
<td></td>
<td>Chemicon, USA</td>
</tr>
<tr>
<td>Rabbit anti-DCX</td>
<td>Neuroblast</td>
<td>1:500</td>
<td></td>
<td>Chemicon, USA</td>
</tr>
<tr>
<td>Goat anti-DCX</td>
<td>Neuroblast</td>
<td>1:2000</td>
<td></td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>Mouse anti-MAP2ab</td>
<td>Neuron</td>
<td>1:500</td>
<td></td>
<td>Sigma, Germany</td>
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<tr>
<td>Mouse anti-NeuN</td>
<td>Neuron</td>
<td>1:500</td>
<td></td>
<td>Chemicon, USA</td>
</tr>
<tr>
<td>Mouse anti-MBP</td>
<td>Oligodendrocyte</td>
<td>1:500</td>
<td></td>
<td>Sternberger Monoclonals Incorporated, USA</td>
</tr>
<tr>
<td>Mouse anti-TGF-bRII</td>
<td>TGFb-signaling</td>
<td>1:50</td>
<td>1:100</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>Rabbit anti-TGF-bRI</td>
<td>TGFb-signaling</td>
<td>1:50</td>
<td>1:1000</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>Rabbit anti-pSmad2(Ser465/467)</td>
<td>TGFb-signaling</td>
<td>1:50</td>
<td>1:1000</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Mouse anti Smad2</td>
<td>TGFb-signaling</td>
<td></td>
<td>1:1000</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Rabbit anti-pCREB(Ser133)</td>
<td>CREB-signaling</td>
<td>1:100</td>
<td>1:1000</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Rabbit anti-CREB</td>
<td>CREB-signaling</td>
<td></td>
<td>1:1000</td>
<td>Cell Signaling, USA</td>
</tr>
<tr>
<td>Rabbit anti-Actin</td>
<td>Loading control</td>
<td>1:5000</td>
<td></td>
<td>Sigma, Germany</td>
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3.1.7. Secondary antibodies

<table>
<thead>
<tr>
<th>List of Antibodies</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-rabbit-HRP</td>
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<td>Dianova, Germany</td>
</tr>
<tr>
<td>Goat anti-mouse HRP</td>
<td>1:5000</td>
<td>Chemicon, USA</td>
</tr>
<tr>
<td>Donkey anti-rat-BT</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Donkey anti-goat-BT</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Donkey anti-rabbit-Alexa 488</td>
<td>1:1000</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Donkey anti-goat, Alexa 488</td>
<td>1:500</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Donkey anti-mouse- Alexa 488</td>
<td>1:500</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Donkey anti-goat, Alexa 568</td>
<td>1:1000</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Donkey anti-mouse- RHOX</td>
<td>1:1000</td>
<td>Dianova, Germany</td>
</tr>
<tr>
<td>Donkey anti-rabbit - Alexa 568</td>
<td>1:1000</td>
<td>Molecular Probes, USA</td>
</tr>
<tr>
<td>Donkey anti-mouse- Cy5</td>
<td>1:1000</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Donkey anti- guinea pig - Cy5</td>
<td>1:500</td>
<td>Jackson Immuno Research, USA</td>
</tr>
<tr>
<td>Donkey anti- mouse IgM - Alexa 568</td>
<td>1:500</td>
<td>Molecular Probes, USA</td>
</tr>
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</table>

3.1.9. Devices

<table>
<thead>
<tr>
<th>Device</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubing pump</td>
<td>Ismatek SA-Switzerland</td>
</tr>
<tr>
<td>Sliding microtome</td>
<td>Leica, Solms Diagnostic Instruments, USA</td>
</tr>
<tr>
<td>Water bath</td>
<td>GFL, Germany</td>
</tr>
<tr>
<td>Slide Moat</td>
<td>Boekel Scientific, USA</td>
</tr>
<tr>
<td>Orbital Shaker</td>
<td>colonial scientific, USA</td>
</tr>
<tr>
<td>Weighing Balances</td>
<td>Sartorius, Gottingen</td>
</tr>
<tr>
<td>Stereology</td>
<td>Stereoinvestigator, MicroBrightField, Colchester, USA</td>
</tr>
<tr>
<td>Fluorescence Microscope Leica DMR with SPOT Camera</td>
<td>Leica, Wetzlar, Germany</td>
</tr>
<tr>
<td>Confocal scanning laser microscope</td>
<td>Leica TCS-NT, Wetzlar, Germany</td>
</tr>
<tr>
<td>Inverse Fluorescence microscope Olympus IX 70 with Color View documentation system</td>
<td>Olympus, Hamburg, Soft Imaging Systems, Münster</td>
</tr>
<tr>
<td>Equipment Description</td>
<td>Supplier/Location</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Light microscope Olympus CK 30</td>
<td>Olympus, Hamburg</td>
</tr>
<tr>
<td>Photometer Ultrospec 2000</td>
<td>Amersham/Pharmacia Biotech, Freiburg</td>
</tr>
<tr>
<td>Centrifuge 5417 R</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td>Megafuge 1.0 R</td>
<td>Heraeus Instruments GmbH, Germany</td>
</tr>
<tr>
<td>Emax Precision Microplate Reader</td>
<td>Molecular Devices, Union City, CA, USA</td>
</tr>
<tr>
<td>Incubator HERA Cell</td>
<td>Heraeus, Germany</td>
</tr>
<tr>
<td>Hera Safe cell culture hood</td>
<td>Heraeus, Germany</td>
</tr>
</tbody>
</table>

### 3.1.10. Software

- **Adobe Photoshop C2 Version 9.0**: Adobe Systems GmbH, München
- **Adobe Photoshop element 5**: Adobe Systems GmbH, München
- **EndNote 10**: Thompson ResearchSoft, USA
- **GraphPad Prism 5**: GraphPad Software Inc., USA
- **Microsoft Office**: Microsoft Corporation
- **Spot Advanced for Mac OS**: Diagnostic Instruments, USA
- **analySIS® 3.2**: Soft Imaging Systems, Münster, Germany
3.2. Methods

3.2.1. Animals

Two to three months old healthy female Fischer-344 rats (N=5) obtained from Charles River were kept under a normal light-dark cycle of 12 hours and had free access to food and water. Eight and 12 months old rats (male) were obtained from a colony of tgHD and WT littermates that has been established at the central animal facility of the University of Hannover, Germany\textsuperscript{135}. For experiments, offspring derived from generation F10 were used. Presence of the huntingtin transgene in tgHD rats was confirmed by tail-DNA genotyping at the age of 3 weeks. The brains from transgenic mice with inducible neuron-specific expression of TGF-beta1 (tTACamKIIa/tTA-responsive promoter (Ptet) TGF-beta1)\textsuperscript{148} were obtained from the Department of Neuroanatomy, University of Leipzig, Germany. Induction of TGF-beta1 expression in these animals was achieved by omitting doxycycline from the drinking water for 54 days (TGF-beta1-on mice; N=4 and TGF-beta1-off mice; N=4). Tissues from R6/2 mice\textsuperscript{149} and from TGF-beta infused rats \textsuperscript{82} were also used in the present study. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local governmental commission for animal health.

3.2.2. Intracerebroventricular infusions of TGF-beta1

Two to three months old (180 g) female Fischer-344 rats (n=16) received intracerebroventricular infusions via stainless steel canules connected to osmotic minipumps (Model 2002, ALZET, Durect Corp., Cupertino, USA) as described\textsuperscript{82}. 
Rats received either recombinant TGF-beta1 (500 ng/ml present in the pump) dissolved in artificial cerebrospinal fluid (aCSF) or aCSF as control (n=8 each) at a flow rate of 0.5 µl/hr for a period of 14 days. During the last four days of the pump period, rats received daily intraperitoneal injections of 50 mg/kg BrdU. Then, rats were intracardially perfused with 4% paraformaldehyde.

3.2.3. BrdU labelling

Labelling of dividing cells was performed by intraperitoneal injection of the thymidine analogue BrdU (5-bromo-2-deoxyuridine) (Sigma, Steinheim, Germany) at 50 mg/kg of body weight using a sterile solution of 10 mg/ml of BrdU dissolved in a 0.9% (w/v) NaCl solution. For proliferation studies, animals received two BrdU injections with an interval of 12 h and were sacrificed 24 hours after the second BrdU pulse. To address cell survival BrdU injections were performed daily on 5 consecutive days (day 1 to 5) and the animals were sacrificed at day 30.

3.2.4. Tissue processing and Immunohistochemistry

Rats or mice were deeply anesthetized using a ketamine (20.38 mg/ml), xylazine (5.38 mg/ml) and acepromazine (0.29 mg/ml) mixture. Transcardial perfusion was performed with 0.9% (w/v) NaCl solution followed by a 4% paraformaldehyde, 0.1 M sodium phosphate solution (pH 7.4). Brains were removed and post-fixed in paraformaldehyde overnight at 4 °C. Tissue was then cryoprotected in 30% (w/v) sucrose, 0.1 M sodium phosphate solution (pH 7.4). Brains were cut into 40 µm sagittal sections using a sliding microtome on dry ice. Sections were stored at - 20 °C in cryoprotectant solution (ethylene glycol, glycerol, 0.1 M phosphate buffer pH 7.4, 1:1:2 by volume). Free-floating sections were treated with
0.6% H\textsubscript{2}O\textsubscript{2} in Tris-buffered saline (TBS: 0.15 M NaCl, 0.1 M Tris–HCl, pH 7.5) for 30 min. For immunohistological detection of the incorporated BrdU, pre-treatment of tissues was performed as described previously\textsuperscript{150}. Following extensive washes in TBS, sections were blocked with a solution composed of TBS, Triton X 100, 0.1%, bovine serum albumin 1%, and teleostean gelatine (Sigma, Taufkirchen, Germany) 0.2% for 1 h. This buffer was also used during the incubation with antibodies. Primary antibodies were applied overnight at 4°C. For chromogenic immunodetection, sections were washed extensively and further incubated with biotin-conjugated species-specific secondary antibodies followed by a peroxidase–avidin complex solution from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA). The peroxidase activity of immune complexes was revealed with a solution of TBS containing 0.25 mg/ml 3, 3’-diaminobenzidine (DAB) (Vector Laboratories, Burlingame, USA), 0.01% (v/v) H2O2, and 0.04% (w/v) NiCl2. Sections were put on Superfrost Plus slides (Menzel, Braunschweig, Germany) and mounted in Neo-Mount (Merck, Darmstadt, Germany). For epifluorescence immunodetection, sections were washed extensively and incubated with fluorochrome-conjugated species-specific secondary antibodies for overnight at 4°C. Sections were put on slides and mounted in Prolong Antifade kit (Molecular Probes, Eugene, USA). Photodocumentation was done using a Leica microscope (Leica, Wetzlar, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, Sterling Heights, USA) and epifluorescence observation was performed on a confocal scanning laser microscope (Leica TCS-NT, Wetzlar, Germany).

The following antibodies and final dilutions were used. Primary antibodies: rat anti-BrdU 1:500 (Oxford Biotechnology, Oxford, UK), mouse anti-PCNA (Proliferating Cell Nuclear Antigen) 1:500, goat anti-DCX (Doublecortin) (C-18) 1:500 (both from
3.2.5. Counting procedures

All morphological analyses were performed on blind-coded slides. Every sixth section (240-µm interval) of one hemisphere was selected from each animal and processed for immunohistochemistry. To analyze cell proliferation in the dentate gyrus (DG) (experiment I), BrdU and PCNA immunopositive cells were counted. To assess cell survival in DG (experiment II) BrdU immunopositive cells were quantified. To investigate neurogenesis, the number of neural precursor cells ever 12th section (480 µm interval) stained for DCX was determined. All cells that were stained by BrdU, PCNA or DCX antibodies were counted with 400 x magnification on a light microscope (Leica, Wetzlar, Germany) and multiplied by 6 or 12 to obtain an estimate of the total immunopositive cell numbers. The reference volume was determined by tracing the granule cell layer of the hippocampal DG using a semi-automatic stereology system (Stereoinvestigator, MicroBrightField, Colchester, USA). The total number of neurons in DG was determined in every 12th section (480µm intervals) stained for NeuN. Positive cells for NeuN in the granule cell layer of the dorsal hippocampal dentate gyrus were analysed. A systematic counting
procedure, similar to the optical dissector described by Gundersen et al.,\textsuperscript{151} and Williams & Rakic (1988).\textsuperscript{152} NeuN positive cells were counted within a 15 x 15 µm counting frame that was spaced in a 150 x 150 µm counting grid. Granule cell nuclei intersecting the uppermost focal plane (exclusion plane) and those intersecting the exclusion boundaries of the counting frame were not counted. The sample volume of NeuN positive cells was determined by multiplication of number of frame counted x size of the counting frame x thickness of the section. Volume of the entire structure is derived by multiplying the reference volume with serious of sections. To obtain the neuronal density the total number of cells counted is divided sample volume and represented as cells/mm\textsuperscript{3}. All extrapolations were calculated for one hippocampus and should be doubled to represent the total hippocampal values. We noticed that BrdU positive cells appeared often in clusters, which can be considered as proliferating units.\textsuperscript{153} To analyze proliferating units, size of clusters (> 3 cells) in µm\textsuperscript{2} and number of BrdU cells per clusters (proliferating units) were quantified on an Olympus microscope (Olympus, Hamburg, Germany) using analySIS\textsuperscript{®} 3.2 software (Soft Imaging Systems, Münster, Germany). For the analysis of mitotic neuronal precursor cells, the BrdU/DCX double positive cells were analysed in BrdU positive cell clusters 24 h after BrdU injection.

To determine the frequency of neuronal differentiation and cell fate of newborn cells (experiment II), a series of every sixth brain section (240-µm interval) was stained and analyzed for BrdU/NeuN/GFAP by triple immunofluorescence. Undifferentiated, self-renewing and quiescent cells were identified by BrdU/Sox2/PCNA triple immunofluorescence. TGF-beta1 signaling was identified by presence of pSmad2 in GFAP+/Sox2+ and in DCX+/NeuN+ cells. Neuronal differentiation was analyzed by BrdU/NeuN double-stainings, astroglial fate was
identified by BrdU/GFAP double-labelling. Stainings were examined using a Leica TCS-NT confocal laser microscope (Leica Microsystems, Bensheim, Germany) equipped with a 40× PL APO oil objective (1.25 numeric aperture) and a pinhole setting that corresponded to a focal plane of 2 µm or less. Due to the relatively high age of the animals and the overall low level of neurogenesis, every BrdU labelled cell was examined in the cell fate analysis. For the determination of TGF-beta1 signaling in neural stem cells or neurons, 50 Sox2 or Sox2/GFAP double positive cells or DCX (immature) or DCX (mature) or NeuN positive cells were examined for pSmad2 co-localization.

3.2.6. Western Blotting

Different brain regions from WT or HD rats, or neurospheres were lysed in buffer (0.7% NP40, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 250 mM NaCl, 10% glycerol, 0.2 mM Na3VO4, 1 mM PMSF, 10 mM DTT, 2µg/ml Aprotinin and 1µg/ml Pepstatin) and centrifuged at 17900 xg for 15 min at 4 °C. The protein concentration was determined using BCA test (Sigma, Taukirchen, Germany) the resulting supernatants (20 µg of total protein) were size-separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by semidry electroblot method (Biometra Fast blot, Biometra Biomedizinische Analytik, Göttingen, Germany). To determine the endogenous level of TGF-beta1 signaling in the rat brain, antibodies used for Western blot and their dilutions were as follows: rabbit anti-phospho Smad2 (Ser465/467) 1:1000 and mouse anti-Smad2 1:1000 (Both from Cell Signaling, USA); mouse anti-TGFbRII, 1:500; Rabbit anti-TGFbR1, 1:1000 (Both from Santa Cruz Biotechnology, USA); Rabbit anti-Actin 1:5000 (Sigma, Germany). Level of CREB signaling was detected
by using rabbit anti-phospho CREB1 (Ser133) 1:1000 and rabbit anti-CREB 1:1000 (Both from Cell Signaling, USA), rabbit anti-Actin 1:5000 (Sigma, Taufkirchen, Germany). Secondary goat anti-mouse 1:5000 (Chemicon, Temecula, USA) or anti-rabbit IgG-HRP antibodies 1:10,000 (Dianova, Hamburg, Germany) and detection was performed using the ECL plus chemiluminescence system (Amersham Pharmacia, Freiburg, Germany) and exposed to Hyperfilm (Amersham Pharmacia, Freiburg, Germany).

3.2.7. Neural stem and progenitor cells culture

Adult hippocampal progenitor cells were isolated and cultured as described before\textsuperscript{154}. Briefly, two to four month-old female Fischer-344 rats (Charles River Deutschland GmbH, Germany) were decapitated and hippocampi were dissected. The tissue was homogenized and cells were resuspended in Neurobasal (NB) medium (Gibco BRL, Germany) containing B27 supplement (Gibco BRL, Germany), 2 mM L-glutamine (PAN, Germany), 100 U/ml penicillin / 100µg/ml streptomycin (PAN, Germany). For expansion, the NB/B27 was growth factor-supplemented with 2 mg/ml heparin (Sigma, Germany), 20 ng/ml FGF-2 (R&D Systems, Germany) and 20 ng/ml EGF (R&D Systems, Germany) (proliferation medium). The cell suspension from hippocampi of 5 rats was seeded into a T25 flask (TPP, Switzerland) in 5 mL proliferation medium. The cultures were maintained at 37\degree C in a humidified incubator with 5% CO\textsubscript{2} and half of the media was changed every 3 to 4 days. For passaging, cells were dissociated by using accutase (PAA, Austria) and a total of 5 x 10\textsuperscript{5} cells were seeded in a T75 flask (TPP, Switzerland). Neurosphere cultures from passages 3-7 were used for the experiments.
3.2.8. Cell cycle analysis

The cell cycle was analyzed by fluorescence activated cell sorting (FACS) using a modified Ki67/PI staining protocol from Endl and colleagues\textsuperscript{155}. Neural progenitors derived from adult rat hippocampus were seeded into T25 flasks (TPP, Switzerland) and stimulated with 10 ng/ml TGF-beta1 or PBS control for 7 days. TGF-beta1 was added every second day. On day 7, cells were spun down, medium was removed and cells were fixed with 1 ml ice cold 70% EtOH. After fixation cells were washed with ice cold PBS and then resuspended in 500 µl ice cold PBS containing 0.1% TritonX-100 and incubated for 5 min on ice. After two subsequent washing steps, cells were resuspended in 100 µl of a 1:6 dilution of the antibodies in PBS (100 µl PBS + 20 µl antibody IgG or Ki67; BD Biosciences Pharmingen, FITC conjugated antibodies). After 30-40 min incubation at 4°C, cells were washed with 1 ml PBS and then resuspended in PBS for staining. In case of PI staining, cells were resuspended in 470 µl PBS. Cells without PI staining were resuspended in 495 µl. All samples were treated with 5 µl of RNase for 1 hour to eliminate the RNA, which would interfere with the PI staining. After this 1h incubation at 37°C, 25 µl of PI were added to the respective samples. The stained cells were analyzed with the Flow Cytometer (FACS Calibur, Becton Dickinson, Heidelberg). Data was analyzed using WinMDI 2.8 software.

3.2.9. Immunocytochemistry

Fixed cells were washed in TBS (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5), then blocked with solution composed of TBS; 0.1% Triton-X100 (only for intracellular antigens); 1% bovine serum albumin (BSA) and 0.2% Teleostean gelatin (Sigma,
Taufkirchen, Germany) (Fish Skin Gelatine Buffer, FSGB). The same solution was used during the incubations with antibodies. Primary antibodies were applied overnight at 4°C. Fluorochrome-conjugated species-specific secondary antibodies were used for immunodetection. The following antibodies and final dilutions were used. Primary antibodies: rabbit guinea pig anti-GFAP 1:1000 (Progene, Germany); IgM mouse anti-A₂B₅ 1:200 (Chemicon, UK); mouse anti-rat nestin 1:500 (Pharmingen, U.S.A.); mouse anti-Map 2a+2b 1:250 (Sigma, Germany); mouse anti-Myelin Basic Protein (MBP) 1:750 (SMI-94, Sternberger Monoclonals Incorporated, U.S.A.). Secondary antibodies: donkey ant-mouse, rabbit conjugated with Alexa Fluor® 488 (Molecular Probes, U.S.A.), rhodamine X (RHOX) 1:1000 (Dianova, Germany). In cases of detergent-sensitive antigens (i.e., A₂B₅), Triton X-100 was omitted from FSGB buffer. Nuclear counterstaining was performed with 4’, 6’-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 µg/µl (DAPI; Sigma, Germany). Specimens were mounted on microscope slides using in Prolong Antifade kit (Molecular Probes, U.S.A.). Epifluorescence observation and photodocumentation were realized using a Leica microscope (Leica Mikroskopie und Systeme GmbH, Germany) equipped with a Spot™ digital camera (Diagnostic Instrument Inc, U.S.A.).

3.3. Statistical Analysis

The data are presented as mean values ±SD. Two-way analysis of variance (groups ± age) and Bonferroni post test (tgHD rats) or Student t-test (TGF-beta- on mice) were used for analyzing numbers of PCNA-positive and BrdU-positive cells, numbers of BrdU clusters, numbers of BrdU-positive cells per clusters, sizes of BrdU clusters, total DCX-positive cells, total neuronal number and neuronal density, and
percentage of BrdU/NeuN, BrdU/NeuN/GFAP- and BrdU/Sox2/PCNA-positive cells. One-way analysis of variance test was used for percentage of Sox2/GFAP/pSmad2-positive cells, and a Tukey test comparison was performed for post hoc analysis. Student t-test was applied for analyzing the number of PCNA-positive cells in R6/2 mice, percentage of BrdU/DCX double-positive cells in tgHD and WT rats, numbers of BrdU-positive cells in the TGF-beta1 infusion study, and for cell cycle phase analysis. Statistical analysis was performed using Prism (Prism GraphPad Software, San Diego, CA). The significance level was at \( p \leq 0.05 \), unless otherwise indicated.
4. Results

4.1. TGF-beta1 signaling components are expressed throughout the adult rat brain.

TGF-b receptor II, receptor I, and Smad2 are integral downstream constituents of the TGF-beta1 signaling cascade. Typically, ligand binding and TGF-beta receptor activation triggers phosphorylation of Smad2, and phospho-Smad2 is widely used as a marker indicating a TGF-beta signaling response. Here, we investigated protein expression of TGF-bRII, TGF-bRI, Smad2 and phosphorylation of Smad2 in the hippocampus (HC), ventricle wall including the sub-ventricular zone (SVZ), olfactory bulb (OB), cortex (Cor) and cerebellum (CB) by Western blot analysis; lung tissue was used as control (Fig 4.1). TGF-betaRII and RI were detected in all brain regions with the neurogenic regions HC and SVZ showing the least expression. The highest expression of TGF-betaRII was found in the lung. In contrast to that, the downstream signaling molecule Smad2 was strongly expressed in the different brain regions and only faintly detected in the lung. More importantly, while in lung tissue Smad2 was apparently in its non-phosphorylated and therefore inactive state, it was phosphorylated in the brain. This suggests that TGF-beta signaling is active in various regions of the adult rat brain including the neurogenic regions (Fig 4.1).
Fig. 4.1. Western blot analysis demonstrating TGF-beta1 signaling components in intact adult brain. Note the expressions of TGF-betaRII, TGF-betaRI and phosphorylated form of Smad2 in different brain regions. B-Brain, HC-Hippocampus, SVZ-Sub ventricular Zone, OB-Olfactory bulb, Cor-Cortex, CB-Cerebellum and non-neuronal tissue- Lung. Smad2 and Actin as controls.

In order to substantiate the TGF-beta signaling profiling, we performed an extensive semiquantitative immunohistochemical analysis of the TGF-beta signaling components in different brain regions. Overall, we encountered a low immunoreactivity of TGF-betaRII in the adult rat brain (Table 1 and Fig 4.2). Faint perinuclear signaling was present in cells of the CA1 and CA2 regions of hippocampus, in the cerebral cortex cortex, in Purkinje cells of the cerebellum and cells of the brain stem (Table 1). Within the neurogenic regions TGF-betaRII immunoreactivity was faint (Table 1, Fig 4.2). In contrast to the weak TGF-betaRII immunoreactivity, a robust staining for TGF-betaRI was noticed in the HC, in the rostral migratory stream (RMS) and in the OB. In the stem cell niche, expression of TGF-betaRI was clearly visualized in the SGZ of HC and in the SVZ. In addition, expression of TGF-betaRI was found in cells of the striatum, Cor, CB and in the brain stem (Table 1 and Fig 4.3). A prevalent expression of pSmad2 was observed in most brain regions (Table 1 and Fig 4.4). For example, pSmad2 immunoreactivity was
prominent in the Cor, Strr, CB and in the brain stem, in the CA1, CA2 and CA3 regions of HC, in the SVZ, along the RMS and in the OB.

**Fig. 4.2.** Localization of TGF-betaRII (green) immunoreactivity in different areas of the intact adult rat brain. Note the expression of TGF-betaRII in the DG-Dentate Gyrus, in the SVZ-Subventricular Zone, in the OB-Olfactory bulb and in the Cor-Cortex. scale bar, 50µm. Insets are higher magnifications of selected fields.
Fig. 4.3. Localization of TGF-betaRI immunoreactivity in different areas of intact adult rat brain. Note the widespread expression of TGF-betaRI in the DG-Dentate Gyrus, in the SVZ-Subventricular Zone, in the OB-Olfactory bulb, in the Str-Striatum, in the Cor-Cortex, in the CB-Cerebellum and in the B Stem-brain stem. scale bar, 100 µm. Insets are higher magnifications of selected fields.
Fig. 4.4. Localization of pSmad2 immunoreactivity in different areas of intact adult rat brain. Note the phosphorylated form of pSmad2 expression in the DG-Dentate Gyrus, SVZ-Subventricular Zone, OB-Olfactory bulb, Str-Striatum, Cor-Cortex, CB-Cerebellum and B Stem-brain stem. However, it is highly limited in stem cell niche, SGZ of DG and SVZ. scale bar, 100µm. Insets are higher magnifications of selected fields.
4.2. Table 1

Semiquantitative measurement of immunoreactivity of TGF-betaRII, TGF-betaRI and pSmad2 in the adult rat brain

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>TGF-betaRII</th>
<th>TGF-betaRI</th>
<th>pSmad2</th>
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<tr>
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<td>Olfactory system</td>
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<tr>
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<tr>
<td>Granule cell layer</td>
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<td>++++</td>
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<tr>
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<td>++++</td>
</tr>
<tr>
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<td>Substantia nigra pars reticulate</td>
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<tr>
<td>Rostral Migratory Stream (RMS)</td>
<td>&lt;+</td>
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</table>

**Table 1.** Each brain region was evaluated for the relative density of TGFβRII, TGFβRI or pSAMD2 immunostaining. (−) no stained cells observed, (+) only few
cells stained, (++) sparse staining (stained cells in < 25% field), (+++) strong staining (stained cells in <50% field), (++++) robust staining (stained cells/fibers in >50% field).

4.3. **pSmad2 is predominantly present in postmitotic cell in the hippocampus of the adult rat brain**

For a more detailed investigation on the expression of pSmad2 in the hippocampal neurogenic niche we analyzed neural stem cells (radial GFAP and/or Sox2 expressing cells), young immature neurons (DCX expressing cells), and mature neurons (NeuN positive cells) for the presence of pSmad2 immunoreactivity. Most of the GFAP positive cells within the SGZ (77.4 ± 4.1%) were negative for pSmad2 immunoreactivity (Fig 4.5). Likewise, the majority of GFAP/Sox2 double positive cells (62±2%) in the SGZ failed to co-localize with pSmad2 (Fig 4.5). Approximately half of the Sox2 expressing population (48.6±4.1%) stained for pSmad2. The DCX population was subdivided according to their dendritic morphology\textsuperscript{150} into immature (short horizontal processes) and mature (perpendicular dendritic arborization into the molecular layer) DCX positive cells\textsuperscript{150}. Here, 37.5±7.8% of DCX cells with an immature morphology, and 79.3±5% of DCX-expressing cells presenting a mature morphology showed a positive pSmad2 staining (Fig 4.5). Virtually all (96±2%) NeuN expressing cells stained for pSmad2 (Fig 4.5). The summary of these data is presented in figure 4.5F. Overall, the expression pattern of pSmad2 in the hippocampal neural stem cell niche suggests that TGF-beta signaling is active primarily in cells with neuronal commitment and/or neuronal identity.
Fig. 4.5. Expression of pSmad2 (green) in stem cell niche of the intact adult rat brain.

A) Absence of pSmad2 (green) in GFAP positive (blue) in SGZ. B) Co-localization of pSmad2 (green) in Sox2 (red) positive cells but not in all Sox2 positive cells in SGZ. C) Appearance of pSmad2 (green) in DCX cells of the hippocampal DG. D) Solid co-localization of pSmad2 (green) in NeuN (red) positive neurons in the GCL. Scale bar, 50 µm. Insets are higher magnifications of the selected fields. E) Quantitative analysis of GFAP, Sox2, Sox2/GFAP double, DCX and NeuN labelled cells that are positive for pSmad2. Note the gradual increase of percentage of cell from stem cells towards neurons that co-labelled with pSmad2. F) Schematic summary of pSmad2 expression (green) in neurogenesis at different cellular level in Hippocampal DG. Expression of pSmad2 (green) is prominent in NeuN positive cells (gray cells) and certain amount of DCX expressing cells (red). However, it is absent or low in the GFAP and Sox2 expressing stem cells (blue) in SGZ.
In a parallel study, we noticed that approximately 25% of the BrdU label retaining cells in the hippocampal stem cell niche were Sox2 positive and quiescent, i.e. PCNA negative. Moreover, a minor population of label retaining cells were Sox2 and PCNA positive (less than 5%). This, together with the present findings on pSmad2 positive as well as negative subpopulations of Sox2 and GFAP expressing cells provokes the possibility that stem cell quiescence might be regulated through pathways involving Smad2 signaling. Also, since i) the young immature DCX population of cells is in cell cycle and the mature DCX positive cells are postmitotic ii) pSmad2 in DCX positive cells is found primarily in the mature population, we hypothesized that cell cycle exit in the DCX population might involve Smad2 signaling. Therefore, we analyzed Sox2, GFAP and also DCX expressing cells in the hippocampal stem cell niche for the presence or absence of pSmad2 and/or PCNA. In most of the cases, PCNA and pSmad2 were virtually exclusive. Sox2 positive cells in the SGZ, which were pSmad2 positive, did never label for PCNA and vice versa (Fig 4.6). The vast majority of GFAP positive cells failed to stain for pSmad2. The immature DCX population was pSmad2 negative but positive for PCNA (Fig 4.6). In summary, while proliferating progenitors and neural stem cells are mostly devoid of any detectable pSmad2, it appears that cells become pSmad2 positive while they exit the cell cycle to either progress along the neuronal determination, differentiation and maturation program or to enter a quiescent neural stem cell stage.
Fig.4.6. TGF-beta1 signaling is absent in proliferating stem and progenitor cells A) Absence of pSmad2(green) in Sox2 (blue)/PCNA (Red) double positive cells in the SGZ of the hippocampus but arrow shows co-localization of pSmad2 (green) in Sox2 (blue) positive /PCNA negative cell(red). B) Absence of pSmad2 (green) in GFAP (blue)/PCNA (Red) double positive cells in the SGZ of the hippocampus. C) Absence of pSmad2 (green) in DCX (blue)/PCNA (Red) double positive cells in the SGZ of the hippocampus. Insets are higher magnifications of the selected fields.

4.4. Induced over-expression of TGF-beta1 in the hippocampus reduces cell proliferation, but promotes neuronal differentiation and survival.

Wachs et al., had recently demonstrated that a 7 day intracerebroventricular infusion of TGF-beta1 reduced cell proliferation in the hippocampus and ventricle wall without affecting cell fate. Based on the stimulatory effects of TGF-beta1 on neuronal differentiation, functional maturation and survival presented above, we re-examined the effects of TGF-beta1 on the neural stem cell niche, however, using an animal model that allows the inducible and sustained expression of TGF-beta in the hippocampus. This was accomplished using our previously generated transgenic mouse model, which expresses TGF-beta1 in the hippocampus under a tetracycline regulatable Ca-Calmodulin kinase promoter.
First, we confirmed that a 2 months induction of TGF-beta1 elevates the levels of pSmad2 in the dentate gyrus. In addition to the overall elevation of the staining intensity of pSmad2 in the granule cell layer, the TGF-beta-on mice presented strong pSmad2 immunoreactivity in Sox2 / GFAP positive cells, compared to the TGF-beta-off animals (Fig. 4.7a). Cell proliferation, as determined by the number of PCNA positive cells, in the subgranular zone was significantly reduced in the TGF-beta-on mice (Fig. 4.7b). The number of young immature neurons, however, as determined by counting of DCX positive cells, was not changed (Fig. 4.7b). Moreover, the number of surviving cells 4 weeks after BrdU labelling, was increased (Fig. 4.7b). Thus, even though less cells are produced after TGF-beta1 over-expression, the survival of newly generated cells is dramatically increased resulting in a net gain of cells. Even though the percentage of cells acquiring a neuronal phenotype (approx. 60%) did not change upon TGF-beta1 over-expression, the increased survival of cells results in a net gain of neurons in the hippocampal DG (Fig. 4.7b).

![Fig. 4.7a. pSmad2 staining in hippocampal DG of TGF-beta-off and -on mice. Note the prominent immunoreactivity of pSmad2 in the hippocampal DG of TGF-beta on mice. pSmad2 (green) is strongly present in Sox2 (red)/GFAP (blue) double](image-url)
positive cells specifically in the SGZ of the hippocampus from TGF-beta1-on mice. Scale bar, 100 µm.

**Fig.4.7.b. Reduced proliferation but increased neuronal survival in the hippocampus of TGF-beta-on mice**

A-A') Quantification of the number of PCNA positive cells in the DG of TGF-beta-off and TGF-beta-on mice. Data are expressed as means ± SD. For statistical analysis a Student t-test was performed. Note the reduction of PCNA positive cells in TGF-beta-on mice (A' p<0.05). B-B') Quantification of the number of DCX positive cells in the DG of TGF-beta-off mice and TGF-beta-on mice and result are not significant (B'). C-C') Quantification of the number of BrdU positive cells (from 4 weeks of survival experiment) in the DG of TGF-beta-off mice and TGF-beta-on mice. Note the increment of BrdU positive cells in TGFbeta-on mice (C' p<0.01). D-D') Quantification of the phenotype of BrdU positive cells. Note the increased total number of BrdU/NeuN double positive neurons in the DG of TGF-beta-on mice.
(D^2; p<0.05). Scale bar, 50 μm. Insets are higher magnifications of the selected fields.

4.5. Elevated levels of TGF-beta1 provokes expression of pSmad2 in neural stem and progenitor cells.

TGF-beta signaling seems to be absent or low in the proliferating neural progenitor cell population, while in neighboring quiescent stem cells and neurons the TGF-beta signal cascade is on. Thus, we wanted to know, if a TGF-beta response can be triggered in the neural stem and progenitor population by elevated levels of TGF-beta1. In vitro, expression of TGF-betaRII, TGF-betaRI and pSmad2 was evaluated by Western blot analysis and by immunofluorescence stainings.

TGF-betaRI and Smad2 were clearly detected in control as well as in TGF-beta1-stimulated neurosphere homogenates (Fig 4.8). TGF-betaRII was weakly present in control cultures, however, elevated after TGF-beta1 stimulation with a maximum expression level at 2 hrs after treatment (Fig 4.8). pSmad2 was below the level of detection in control neurosphere homogenates, but induced after TGF-beta1 stimulation with a peak level of phosphorylation at 2 hrs after stimulation (Fig 4.8). Since neurosphere cultures consist of a heterogeneous cell population with stem cells, progenitors and determined cells present in the cultures, we analyzed the identity of neurosphere-derived cells that respond to TGF-beta1 with an induction of a pSmad2 signal under proliferation conditions, i.e. in the presence of EGF and FGF-2. While GFAP positive, Nestin positive and A₂B₅ positive cells (Fig 4.9) were virtually devoid of any detectable pSmad2 signal in the control conditions, TGF-beta1 induced the appearance of a nuclear pSmad2 signal 90 min after TGF-beta1 stimulation indicating that neural stem and progenitors are responsive to TGF-beta1 (Fig 4.9). As one of the biological consequences of TGF-beta1 signaling in
neurosphere cultures, we recently demonstrated that TGF-beta1 stimulation inhibits progenitor proliferation \(^8_2\).

**Fig.4.8. Western blot analysis demonstrating the kinetic regulation of TGF-beta1 signaling in stem and progenitor cells in vitro.** Note the trace amount of expressions of TGF-beta RII in NSC-NPCs, it is slightly up regulated at 80 mins and 2hr upon TGF-beta1 stimulation while expression of TGF-bRI showed a steady state level. However, Smad2 is not phosphorylated in NSC-NPC, but it peaked at 80 mins and 2 hrs upon TGF-beta1 stimulation. Even though it is tent to baseline, phosphorylation of pSmad2 is detectable after 12 hrs that show its long lasting capacity.
Fig. 4.9. Immunostaining of pSmad2 in stem cell and progenitor cells in vitro with or without TGF-beta1 treatment. A) Note the translocation of pSmad2 (green) into nucleus (blue-DAPI) A) of GFAP positive cells (red), B) of Nestin positive cells (red) and C) of A2B5 cells (red) in TGF-beta1 treatment, while control condition show a trace amount of pSmad2 expression. Scale bar, 50 µm. Insets are higher magnifications of arrowed fields.
Finally, we used neurosphere derived cells that were differentiated using growth factor withdrawal and addition of serum and analyzed astroglial (GFAP positive), oligodendroglial (MBP positive) and neuronal (Map2ab positive) cells for their responsiveness to TGF-beta1. A 90 min stimulation with TGF-beta1 induced an elevation and translocation of pSmad2 into the nucleus of GFAP positive, MBP positive and Map2ab positive (Fig 4.10) cells indicating that all three cell lineages of the CNS are responsive to TGF-beta1. We also noticed a reduction in the MBP signal in the TGF-beta1 stimulated cultures. This confirms own data demonstrating a significant reduction of MBP mRNA expression in neurospheres after TGF-beta stimulation in a whole genome expression profiling (data not shown).
Fig. 4.10. Immunostaining of pSmad2 in matured cell populations of the CNS at invitro status with or without TGF-beta1 treatment. Note the translocation of pSmad2 (green) into nucleus (blue-DAPI) A) of GFAP positive astrocytes (red), B) of MBP positive oligodendrocytes (red) and C) of MAP2ab positive neurons (red) in TGF-beta1 treatment, while control condition show a basal amount pSmad2 expression. Note the down regulation of MBP expression in oligodendrocytes in TGF-beta1 treatment. Scale bar, 50 µm. Insets are higher magnifications of nucleus of the cells, pSmad2(green) DAPI(blue).

4.6. Regulation of hippocampal neurogenesis in tgHD rats

Cognitive impairment is a prominent manifestation observed in HD patients, as well as animal models of HD\textsuperscript{74, 132, 156, 157.} In the tgHD rat model, working memory deficits starts to develop with 9 months and reaches a plateau with 12 months of age\textsuperscript{132}. Given that working memory is tightly associated with hippocampal function and requires persisting neurogenesis\textsuperscript{158-161}, we hypothesized that the hippocampal stem cell niche in tgHD animals might undergo some fundamental molecular and cellular changes affecting neurogenesis.

4.7. Hippocampal cell proliferation in tgHD rats gets progressively impaired between 8 and 12 months of age.

Neural progenitor’s proliferation in the dentate gyrus (DG) of tgHD rats and WT littermates was analyzed by quantifying the number of BrdU- and of PCNA-positive cells (Fig. 4.11). Eight months old tgHD rats showed a frequency of BrdU incorporation similar to their WT counterpart (WT: 159±78 vs. HD: 179±93) (Fig. 4.11). In contrast, in 12 months old rats the number of BrdU labelled cells in the DG was significantly reduced compared to WT (WT: 88±62 vs. HD: 32±15) (Fig. 4.11). Immunohistological analysis of PCNA, a protein expressed throughout the cell cycle, confirmed this significant reduction in proliferating cells in 12 months old tgHD rats (WT: 99±26 vs. HD: 60±24) (Fig. 4.11). In addition, we also observed the expected
age-related decline in progenitor proliferation in WT controls, although it is assumed to be not reached the level of statistical significance (Fig. 4.11).

Fig.4.11. Impaired hippocampal proliferation in 12 month-old tgHD rats. BrdU labelling of 12 month-old WT and tgHD hippocampus (A). Insets are higher magnifications of the selected fields. Scale bar, 100 μm. Quantitative analysis of number of BrdU positive cells in 8 months (B) and 12 months (C) rats 24 h after BrdU injections and of PCNA expressing cells in 8 months (D) and 12 months (E) rats expressed as positive cells per hippocampus (mean±SD; Student t-test). Note that in the 8 months rats there was no difference between WT and tgHD, while in 12 month-old tgHD the number of BrdU (C) (*p<0.05) and PCNA (E) (*p<0.05) positive cells were significantly reduced. N=4 for WT and N=8 for tgHD.

4.8. Impaired survival of newly generated cells and reduced neuronal density was mediated by reduced CREB signaling in tgHD rat hippocampus.

Next, we analyzed survival and fate of newly generated cells in the transgenic rats four weeks after proliferating cells were labelled with BrdU (Fig. 4.12). The total number of BrdU positive cells was significantly lower in the DG of tgHD rats

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compared to WT in the 8 months (WT 91.5±41 vs. HD 14.2 ± 23; Fig 4.12) and 12 months groups (WT 137 ± 82 vs. HD 53 ± 25; Fig 4.12) indicating that survival of newly generated cells in the DG of tgHD rats was reduced as compared to WT littermates.

Signaling via the cAMP responsive element-binding protein (CREB) is known to be involved in regulating neuronal survival\textsuperscript{162,163}. Thus, we hypothesized that the reduced survival of newly generated cells observed in tgHD rats might correlate with altered CREB signaling. Western blot analysis demonstrated reduced expression of CREB protein in the hippocampi of tgHD rats compared to WT (Fig. 4,12). Moreover, the levels of CREB phosphorylation were diminished in tgHD rats (Fig. 4.12D). Further evidence that CREB signalling is impaired in HD was provided by our recent gene expression profiling of human HD brain revealing a down-regulation of CREB-regulated transcription co-activator 1 mRNA expression as well as of p300/CBP-associated factor and of cyclic AMP-regulated phosphoprotein expression\textsuperscript{71}. Moreover, difference in proliferation of neural progenitors and cell survival in tgHD rat brain might be crucially influenced to total number of existing matured neuron population. In order to test that, we analyzed total number of neuron in the hipocampal DG of transgenic HD rats by using histological brain sections stained for NeuN. The density of total number of NeuN positive cells (cells/mm\textsuperscript{3}) was significantly lower in the DG of tgHD rats compared to WT in the 8 months (WT 4372718±632610.1 vs. HD 3049741±814185; Fig. 4.12) and 12 months groups (WT 3160326 ± 237335 ± 82 vs. HD 2369196 ± 213716 Fig. 4.12) indicating that total number of matured neurons in the DG of tgHD rats was reduced as compared to WT littermates.
Fig. 4.12. Reduced cell survival in tgHD rats is associated with diminished CREB signaling.
A) BrdU labelled cells in the DG 4 weeks after injecting BrdU into 8 month-old WT and tgHD rats; scale bar, 50 µm. Insets are higher magnifications of the selected fields. B) and C), quantitative analysis of BrdU labelled cells 4 weeks after BrdU labelling. Note the significant reduction of surviving cells in the tgHD in the 8 months (B) (**)p<0.001) and 12 months (C) (*p<0.05) (mean±SD; Student t-test). N=4 for WT and N=8 for tgHD. D) Western blot analysis demonstrating lower levels of CREB and p-CREB in tgHD hippocampi. Actin is used as loading-control. E) and F), quantitative analysis of density of NeuN positive cells in the DG. Note the significant reduction of neuronal density in the tgHD in the 8 months (E) (*p<0.05) and 12 months (F) animals (**p<0.0001). G) and H) quantitative analysis of the total number of neurons in the DG. Note the significant reduction in the total number of neurons in the tgHD in 8 months (G) (**)p<0.01) and 12 months (H) animals (**p<0.0001). Data are expressed as means ± SD. For statistical analysis a student test was performed. N=4 for WT and N=8 for tgHD in E-H.
4.9. Increased quiescence of newly generated cells in tgHD dentate gyrus.

Four weeks after BrdU labelling, neuronal and astroglial differentiation of newly generated cells was analyzed by confocal analysis of BrdU labelled cells positive for NeuN (neurons) or GFAP (astroglia) expression (Fig. 4.13). In the DG of 8 and 12 month-old WT rats, newly generated cells predominantly differentiated into neurons (Fig. 4.13). In tgHD rats in contrast, neuronal differentiation was significantly reduced (8 months: WT, 77.7±6.6 vs. HD, 30.3±7.9%; 12 months: WT, 76.5±21 vs. HD, 43.8±13.6%) (Fig. 4.13). Regardless of the age and the presence of the transgene, approximately 10% of newly generated cells differentiated into GFAP-positive astroglial cell (data not shown). However, there was a striking increase in the pool of BrdU-positive cells that did neither co-label with NeuN nor with GFAP (8 month: WT, 11.9 ± 4 vs. HD, 57.8 ± 16.5%; 12 month: WT, 15.9 ± 4.7 vs. HD, 48.9 ± 22%). Thus, reduced neuronal differentiation of newly generated cells was associated with an increase of cells remaining undifferentiated and/or in a quiescent stem cell stage.

To define the cell identity of BrdU-retaining undifferentiated cells in more details, we further analyzed for the presence of Sox2, a marker for functional stem cell maintenance and for the presence of the proliferation marker PCNA. As compared to WT, tgHD rats contained a strongly increased number of Sox2 positive cells in the DG. Moreover, the number of BrdU/Sox2 double positive cells was also significantly increased in the tgHD rats (Fig 4.13) (8 months: WT: 25 ± 1.6 vs. HD: 52.5 ±6.3%; 12 months: WT: 26.3 ± 9.7 vs. HD: 61.62 ± 4%). In both genotypes however, the vast majority of BrdU-retaining/Sox2-positive cells found in the SGZ
were PCNA negative (Fig. 4.13). Taken together, our observation indicates that the number of quiescent stem cells is increased in tgHD hippocampus.

Fig.4.13. Deficiency in neuronal differentiation and augmentation of quiescence in stem cells in tgHD rats.

A) Confocal analysis of BrdU labelled cells (red) that colocalize with NeuN (green) or with GFAP (blue) 4 weeks after BrdU injection of a 8 month-old WT rats. Scale bar = 50µm. B) and C) Quantitative analysis of BrdU labelled cells that did not co-localize with a differentiation marker, or that co-localized with NeuN for neuronal differentiation 8 month-old (B) and 12 month-old (C) rats. Note the reduction in neuronal differentiation that is accompanied with an increase in the number of undifferentiated cells (data presented as means±SD; ***p < 0.0001; *p < 0.05; Two-way analysis of variance-Bonferroni post test). D) Sox2 staining in the DG of WT and tgHD rats (12 months). Note the prominent immunoreactivity of Sox2 in tgHD DG; scale bar, 100µm. E) Illustration of triple staining of BrdU (green) / Sox2 (red) / PCNA (blue) in WT and tgHD DG 4 weeks after BrdU injection (8 Months). Note that in WT, BrdU positive cells are negative for Sox2 and for PCNA, while in tgHD brains, BrdU labelled cells are positive for Sox2 but negative for PCNA. A control for PCNA is included (P). Scale bar = 50µm. F) and G) Quantitative analysis of BrdU labelled cells that are positive for Sox2 but negative for PCNA. Note that the percentage of BrdU labelled cells that co-labelled with express Sox2 and that are PCNA negative is increased in tgHD in 8 months (***p<0.0001) (F) 12 month (**p<0.0001) rats (G). There was no significant difference observed in BrdU/Sox2/PCNA triple positive cells (F, G). N=4 for WT and N=8 for tgHD.
4.10. Neuroblast proliferation compensates stem cell quiescence in tgHD dentate gyrus

As a consequence of increased stem cell quiescence in tgHD SGZ (Fig. 4.13), one could expect total cell proliferation to be diminished. However, in 8 month-old tgHD DG, the rate of cell proliferation was unaffected (Fig. 4.11). To elucidate this apparent incongruity, we investigated the identity of 8 month-old tgHD DG proliferating cells more in details.

Twenty-four hours after BrdU injection, BrdU-labelled nuclei were predominantly found in cell clusters typically containing a minimum of three and up to 6 cells in the innermost region of the GCL and in the SGZ (Fig. 4.14). The number of such BrdU cell clusters, which can be considered as proliferating units, was significantly reduced in the tgHD DG as compared to their WT counterpart (Fig. 4.14) (8 months: WT: 43.5 ± 13.3 vs. HD: 23.2 ± 10.3; 12 months: WT: 15 ± 6 vs. HD: 4.5 ± 2.7). However, the number of BrdU-labelled cells per unit and, as a consequence, the activity of the proliferating units, were significantly elevated in 8 month-old tgHD rats, but not in 12 month-old rats (8 months: HD: cluster size 369.4± 166 µm², 6.6 cells/cluster; WT: cluster size 115± 115 µm², 4.2 ±1.2 cells/cluster) (Fig. 4.14). Co-labelling of BrdU-positive cells in the clusters with the neuroblast marker DCX revealed a significant increase in the proportion of DCX-expressing cells in 8 month-old rats (WT: 47 ± 5 vs. HD: 73 ± 6.6%) (Fig. 4.14). This increase was also reflected in the total number of DCX-positive cells that expanded by 237.5% in the DG of tgHD rats as compared to WT (Fig. 4.14). Moreover, we could detect DCX-containing axons projecting towards the CA3 regions frequently and specifically in the hilus of 8 month-old tgHD rats (Fig. 4.14). The total number of DCX-expressing cells was significantly reduced by 58.7% in the DG of 12 month-old HD rats as compared to
their WT littermates (Fig. 4.14). This important reduction resulted most likely from a combination of impaired proliferation and reduced survival (Fig. 4.11 and 4.12). Thus, despite the elevated numbers of quiescent Sox2-positive stem cells, the overall proliferation level in the DG was maintained in the DG of 8 month-old tgHD rats through a compensative increased proliferation of the DCX-expressing neuroblast population. In the 12 month-old tgHD rats however, the severe proliferative and survival deficit could not be rectified anymore.
Fig. 4.14. Neuroblast proliferation in 8 month old tgHD hippocampus. 
A) Clusters of BrdU positive cells indicating proliferative units in 8 month-old WT and
tgHD DG 24 hrs after BrdU injection; scale bar, 50µm. Quantitative analysis of (B) 
number of BrdU clusters, (C) size of BrdU clusters, (D) number of BrdU labelled cells 
within a cluster. Data presented as means±SD, Student t-test for statistical analysis. 
Note that in tgHD DG the number of proliferating units is reduced in 8 month-old (*p<0.05) and 12 month-old (**p<0.001) rats, however, size of the clusters (*p<0.05) 
and number of BrdU labelled cells within a cluster (*p<0.05) are increased 
specifically in 8 month-old tgHD rats. E) Quantitative analysis of BrdU labelled cells
in proliferating units that co-localize with DCX for neuroblast proliferation in WT and tgHD DG 24 hr after BrdU injection (8 months). Note the significant increase of BrdU labelled cells expressing DCX in the tgHD DG (**p<0.0001). F) and G) quantitative analysis of the percentage of the total amount of DCX positive cells in WT and tgHD DG in 8 month-old (F) and 12 month-old (G) rats. Note that in tgHD DG percentage of DCX positive cells is significantly enhanced in the 8 month-old rats (**p<0.001), while in the DG of 12 month-old tgHD the percentage of DCX positive cells is significantly reduced (**p<0.001). H) DCX positive cells in 8 month-old WT and tgHD; scale bar, 50µm. Insets are higher magnifications of the selected fields. Note the increased numbers of DCX positive cells and expression of DCX in the axons in the hilar region (H). N=4 for WT and N=8 for tgHD.

4.11. Reduced cell proliferation in tgHD animals correlates with increased TGF-beta1 signaling in hippocampal stem cells

Wachs et al., recently demonstrated that TGF-beta1 impairs neural stem and progenitor cell proliferation, a finding also substantiated by other studies. Moreover, in a comparative transcriptome analysis, we showed that mRNA expression of TGF-beta1, as well as the TGF-beta downstream effector elements Smad2 and Smad4, and the TGF-beta inducible extracellular matrix (ECM) components were elevated in human HD brains. This line of evidence points toward the elevation of TGF-beta signaling in tgHD hippocampus, thereby providing an explanation for the reduced stem cell proliferation. TGF-beta signaling was therefore assessed by immunohistochemistry for phospho-Smad2, an intracellular TGF-beta downstream effector molecule, in the DG. A more specific focus was placed on Sox2 and GFAP/Sox2 double positive neural stem cells located in the SGZ. Confocal analysis for pSmad2 immunoreactivity in the DG of WT rats revealed that pSmad2 staining was weak and confined almost exclusively to NeuN positive cells within the GCL (Fig. 4.15). In addition, DCX-expressing cells presenting a mature morphology, i.e. with extensive dendritic arborization, were also positive for pSmad2, while on the other hand DCX cells localized in the SGZ within clusters or
displaying an immature morphology were negative for pSmad2 (Fig. 4.15). GFAP/Sox2 double-labelled cells in the SGZ were virtually devoid of pSmad2 immunoreactivity (Fig. 4.15).

**Fig. 4.15.** Enhanced TGF-beta1/Smad2 signaling in tgHD rats.
A) Presence of pSmad2 in 8 month-old WT DG granule cells. pSmad2 (green) is present in NeuN (blue) expressing cells and in mature DCX (red) positive cells (DCX cells in the granule cell layer presenting a dendritic tree perpendicular to the SGZ), but not in immature DCX positive cells (cells in clusters with a less pronounced
dendrite); scale bar, 50 µm. Insets are higher magnifications of arrowed fields. B) Presence of pSmad2 (green) in Sox2 (red) / GFAP (blue) double positive cells in 12 month-old tgHD SGZ, but not in WT SGZ; scale bar, 50 µm. Insets are higher magnifications of the selected fields. C) Quantitative analysis of Sox2 and Sox2/GFAP double labelled cells that are positive for pSmad2 in 12 month-old WT and tgHD. Note the significant increase in Sox2 positive cells (***p<0.0001) and of Sox2/GFAP double positive cells (***p<0.0001) that co-localize with pSmad2 in 12 month-old tgHD. Data as means±SD; one-way analysis of variance-Tukey post hoc for statistical analysis. N=4 for WT and N=8 for tgHD. D) Western blot demonstrating increased levels of pSmad2 in tgHD hippocampi. Smad2 and Actin are used as controls.

In contrast, 8 month-old (data not shown) and even more pronounced in 12 month-old tgHD rats, the global pSmad2 staining-intensity was elevated in cells of the GCL (Fig. 4.15). Strikingly, overt pSmad2 immunoreactivity was present in GFAP/Sox2 double-labelled cells of the SGZ of 8 months (data not shown) and even stronger of 12 months tgHD rats (Fig. 5B). In addition to staining intensity, the number of Sox2-positive cells and of Sox2/GFAP- positive cells in the SGZ cells that co-labelled for pSmad2 was significantly enhanced in the tgHD rats (Sox2/pSmad2: WT: 20 ± 2.2% vs. HD: 56 ± 7.7%; Sox2/GFAP/pSmad2: WT: 18.5 ± 1.9% vs. HD: 42.2 ± 8.7%) (Fig. 4.15). Western blot analyses confirmed the global increase in Smad2 phosphorylation in the tgHD hippocampus (Fig.4.1 5).

In order to substantiate our observations on the impact of TGF-beta signaling on the neural stem cell niche in HD pathology, we analysed the hippocampus of R6/2 mice, another well-characterized model for HD\textsuperscript{144}. At 9 weeks of age, cell proliferation in the R6/2 DG was significantly reduced as demonstrated by a decrease in the number of PCNA immunoreactive cells (Fig. 4.16, see also \textsuperscript{149}. This correlated with elevated levels of pSmad2 immunoreactivity in the DG of R6/2 mice as compared to WT mice (Fig. 4.16). Moreover, while there was virtually no pSmad2 labelling in GFAP/Sox2 double-positive cells in the SGZ of WT mice, this putative
stem cell population showed strong immunoreactivity of pSmad2 in R6/2 animals (Fig. 4.16).

**Fig.4.16. Reduced proliferation is associated with elevated TGF-beta1 signaling in R6/2 mice.**
A) Quantitative analysis of the number of PCNA positive cells in the DG of 9 week-old WT and R6/2 mice. Data are expressed as means ± SD. For statistical analysis a Student t-test was performed. Note the reduction of PCNA positive cells in R6/2 DG (**p<0.001). B) pSmad2 staining in the DG of WT and R6/2 animals. Note the prominent immunoreactivity of pSmad2 in R6/2 DG; scale bar, 100µm. C) Co-localization of pSmad2 (green) immunoreactivity in Sox2 (red) and GFAP (blue) double positive cells in the hippocampal stem cell niche of WT and R6/2 mice; scale bar, 100µm. Note that pSmad2 is strongly present in Sox2/GFAP double positive cells specifically in R6/2 mice but not in WT.

Next, we confirmed that an elevated TGF-beta level in the brain per se was sufficient to provoke the appearance of pSmad2 in the hippocampal stem cell niche. To this end, we infused TGF-beta1 into the lateral ventricle of WT rats for 14 days and analyzed cell proliferation and the pattern of pSmad2 staining in Sox2 positive
cells. As we and others reported previously\textsuperscript{82, 91}, TGF-beta1 infusion induced a strong inhibition of proliferation in the hippocampal DG (CSF: 689± 40.6 vs. TGF-beta1: 293±31.8) (Fig. 4.17). This proliferative decline was associated with an increase in the number of Sox2-positive cells (Fig. 4.17). As in naïve WT rats, pSmad2 was weakly but consistently present in cells of the GCL and absent in the Sox2/GFAP-positive cells of aCSF-infused rats (Fig. 4.17). In contrast, the infusion of TGF-beta1 in WT rats enhanced the staining intensity of pSmad2 in the GCL and induced the presence of pSmad2 in Sox2/GFAP double positive cells of the SGZ (Fig.4.17) with a similar pattern as observed in the tgHD rats. Moreover, TGF-beta1 induced a fast appearance of pSmad2 in adult rat-derived neural progenitors in an ex vivo situation (Fig.4.17) indicating that these cells are responsive to TGF-beta1.

Finally, we determined if TGF-beta1 indeed provokes a cell cycle arrest in neural progenitors. For that, we stimulated neural progenitors derived from the adult rat hippocampus \textsuperscript{82} in vitro with TGF-beta1 and analyzed the cell cycle phases by Ki67/PI flow cytometry. This method allows the dissection of G0 and G1, since Ki67 is expressed throughout the cell cycle except in G0\textsuperscript{155}. The quantitative analysis revealed that a 7 day treatment with TGF-beta1 induced a shift to G0 phase of the cell cycle (G0: ctrl, 24.8±3% vs. TGF-beta1, 38,5±2.7%; indicating that TGF-beta1 induces neural progenitors to exit the cell cycle and to become quiescent.
Fig. 4.17. TGF-beta1 directed proliferation and cell cycle arrest is associated with enhanced SMAD2 signaling in stem cell niche.

A) Quantitative analysis of number of BrdU positive cells in the DG of TGF-beta1 and aCSF infused rats. Data is expressed as means ± SD. For statistical analysis a student test was performed. Note that in TGF-beta1 infused rats, the number of BrdU positive cells is significantly reduced (***p<0.0001). B) Sox2 immunoreactivity in the DG of aCSF and TGF-beta1 infused animals. Note the prominent immunoreactivity
of Sox2 in TGF-beta1 infused DG; scale bar, 50µm. C) confocal analysis of Sox2 (red) and GFAP (blue) double positive cells that colocalize pSmad2 (green) in the DG of CSF and TGF beta1 infused animals; scale bar, 50µm. Insets are focused on the selected fields. Note the prominent expression of pSmad2 in Sox2/GFAP double positive SGZ cells in TGF-beta1 infused animals, while CSF infused animals do not present pSmad2 in Sox2/GFAP positive cells. D) Western blot analysis demonstrating TGF-beta1 induced phosphorylation of Smad2 in neural progenitor cultures. Smad2 and Actin as controls. E) Quantitative analysis of Ki67/PI flow cytometry of neural progenitors demonstrating a TGF-beta1 induced cell cycle phase shift into G0 (**p < 0.01). For statistical analysis a student test was performed.
5. Discussion

This thesis addressed the possible role of TGF-beta signaling in the hippocampal stem cell niche of the healthy and the disease brain, in particular of the HD brain. This study provides: 1) a complete profile of the receptor mediated TGF-beta1 signaling in the intact rat adult brain, 2) the evidence for functional role of TGF-beta1 signaling in the stem cell maintenance, neuronal differentiation and neuroprotection in the adult rodent brains and 3) the proof of stem cell quiescence and impaired neurogenesis in the hippocampus of Huntington’s disease animal models which we correlated with enhanced TGF-beta1 signaling.

TGF-beta1 signaling the intact adult rat brain

This study demonstrates that the normal adult rat brain displays an active level of TGF-beta signaling. The immunohistochemistry and Western blotting methods confirmed the expression of TGF-betaRI, TGF-betaRII and phosphorylated status of Smad2 throughout the normal adult rat brain including neurogenic areas. This finding validates previous study that indicated an overall high level of TGF-beta signaling in the brain of SBE luciferase mice\textsuperscript{85}. This suggest the presence of physiological TGF-beta1 levels in most brain areas\textsuperscript{164, 165}.

Role of TGF-beta1 in stem cell maintenance of the adult brain

For the expression of pSmad2 in the hippocampal neurogenic niche, the co-immunostainings and confocal microscopy methods revealed that the NSCs population that are in proliferative phase failed to express pSmad2. However, quiescent stem cells displayed strong pSmad2 signal as indicated by PCNA and pSmad2 co-immunostainings with Sox2 or GFAP. There, the PCNA negative fraction
of Sox2 or GFAP expressing stem cells showed a strong nuclear appearance of pSmad2. An over-expression or infusion of TGF-beta1 in the adult rodent brains inhibited the proliferation of NSCs in the hippocampus and the SVZ.\textsuperscript{82, 91}. This present study also confirmed the previous reports however by using TG TGF-beta-on mice that over-expressed TGF-beta1 in the hippocampus under the tet-regulated system.\textsuperscript{148} A two month periods of TGF-beta1 induction by doxycycline withdrawal (TGF-beta1-on mice) inhibited NSC proliferation as seen by reduced number of PCNA positive cells in the hippocampal DG compared to doxycycline treated animals(TGF-beta1-off mice). Doxycycline withdrawal induced the elevation of pSmad2 in the SGZ Sox2/GFAP expressing NSCs in the hippocampus of TGF-beta-on mice. Thus, a crucial role of TGF-beta1 in the adult hippocampal niche is to control the balance between the quiescent and proliferating NSCs, thereby modulating the stem cell potential and to avoid the stem cell depletion in the stem cell niche of the adult brain. Further, in vitro studies using neurosphere cultures derived from the adult rat hippocampus provided an authentication of receptor mediated TGF-beta1 activation on these cells as demonstrated by Western blot and immunocytochemistry. TGF-beta1 stimulation in neurospheres induced a high level phosphorylation of Smad2 at 2 hrs and increased nuclear translocalization of the pSmad2 including in differentiated stem and progenitor cells. Moreover, one-week incubation of neurosphere cultures with TGF-beta1 inhibited cell proliferation and promoted cell cycle exit. The cell cycle exit is a primary requisite for quiescence and this could be a protective mechanism involved to balance stem cell depletion in the adult brain. It has been reported that TGF-beta1 promotes the cell cycle exit by inducing the expression of the cell cycle protein, p21\textsuperscript{82, 166}. Therefore, TGF-beta1 induced activation of smad2 phosphorylation in the proliferating NSCs would be a
key molecular event to achieve stem cell quiescence in the adult brain. This induced stem cell quiescence could be a consequence of seizing the proliferating NSCs in brain pathologies associated with a prompt elevation of TGF-beta1.

**TGF-beta1 promotes neuronal differentiation and survival in the adult brain.**

In addition to the TGF-beta1 induced anti proliferative effect on NSCs in the adult brain, the present work also demonstrates the involvement of TGF-beta1 in neuronal differentiation and neuronal survival. As observed from co-immunofluorescence staining, TGF-beta signaling is active predominately in cells with neuronal commitment and neuronal identity as indicated by increased percentage of pSmad2 colocalization with DCX and NeuN positive neurons. In the adult stem cell niche while pSmad2 expression is low in the young immature DCX positive cells, pSmad2 localization is increased in the morphologically matured DCX expressing neurons as demonstrated by co immunostainings of PCNA, pSmad2 and DCX with morphological difference. The increased accumulation of pSmad2 from the proliferating DCX sub-population to the morphologically matured DCX positive neurons might be a crucial event for neuronal differentiation and subsequent integration in to neuronal circuit. In consistent nearly all NeuN positive matured neurons in the hippocampal DG displayed a strong signal for pSmad2 in their nucleus. It has been demonstrated previously that newly born DCX expressing cells ultimately turn into NeuN positive neurons in the hippocampal DG of the adult brain\(^{150}\). The analysis of neuronal differentiation in the hippocampus of TGF-beta-on mice indicated that the number of surviving cells 4 weeks after BrdU labelling was increased. Thus, even though a reduced number of cells were generated after TGF-beta1 over-expression, the survival of newly generated cells was dramatically
Neurogenesis and TGF-beta1 signaling in Huntington's diseased brain

In the context of neurodegenerative disease, the present work demonstrates impaired progenitor proliferation associated with an increase in neural stem cell quiescence in animal models for HD (summarized in Fig. 5.1). TGF-beta1 signaling appears to be involved in triggering quiescence of stem cells, as suggested by the presence of pSmad2 in Sox2/GFAP-expressing SGZ cells in tgHD rats, R6/2 mice and after TGF-beta infusion, as well as by the TGF-beta1 induced cell cycle arrest in neural progenitor cultures. In 8 months old tgHD rats, i.e. in the early phase of the pathology, the deficit in stem cell proliferation was compensated by increased DCX-expressing neuroblast proliferation, which resulted in an expansion of the DCX-expressing cell population. Survival of newly generated cells, the total number of dentate gyrus neurons and neuronal density were also reduced in tgHD rats and correlated with weaker pCREB signaling (Fig. 4.12). Similar observations were previously reported for R6/2 mice. Further evidence for a reduced pCREB signalling in HD was provided by expression profiling of human HD brains demonstrating a down-regulation of CREB-regulated transcription coactivator1, of p300/CBP-associated factor and of cyclic AMP-regulated phosphoprotein.
Fig. 5.1. Schematic summary of modulation of neurogenesis at different cellular levels in the transgenic rat model of Huntington disease (tgHD).

In 8-month-old tgHD animals, the deficit in stem/progenitor proliferation was compensated by doublecortin (DCX)-positive neuroblasts (red cells) resulting in an overall increase in the DCX population. In 12-month-old tgHD rats, reduced proliferation of progenitors was accompanied by a reduced number of DCX-positive cells. There were fewer surviving newly generated cells (round-shaped dark blue cells) in 8- and 12-month-old tgHD than in wild-type (WT) animals. The numbers of label-retaining quiescent subgranular zone stem cells, however, were elevated in tgHD animals. In WT, transforming growth factor-beta1 signaling (fluorescence green arrow) was confined to cells of granule cell layer (gray cells) and it was absent or low in the stem cell niche. In tgHD animals, however, the overall TGF-beta1 signaling was greater and prominent in the stem cell niche where it induced cell cycle exit and stem cell quiescence.

Neural stem cell quiescence in neurodegeneration

One of the most striking findings of the present work is the pathology-induced quiescence of neural stem cells in the hippocampal stem cell niche. This quiescence was revealed by an expansion in the pool of label retaining, non-proliferative and undifferentiated cells that expressed in addition the stem cell maintenance marker Sox2. A number of studies have already investigated cell proliferation in the neural
stem cell niche of animal models of neurodegeneration and reported reduced progenitor proliferation rates. Hence, this proliferative decline was documented in the R6/1 and R6/2 HD mouse model\textsuperscript{98, 169, 170} in the A53T mutant alpha-synuclein mouse model of Parkinson's disease\textsuperscript{171}, in a mouse model of Alzheimer's disease, which expresses the human amyloid precursor protein (APP) with the V717F mutation (PDAPP mice)\textsuperscript{172} and in the superoxide-dismutase 1 mouse model of Amyotrophic Lateral Sclerosis\textsuperscript{173}. However, reduced neurogenesis under chronic neurodegenerative disease cannot be taken as a general rule, since cell proliferation in the subventricular zone of human HD brains and hippocampal neurogenesis in human AD brains is enhanced\textsuperscript{147, 174}.

A reduction in the numbers of proliferating cells might result from i) a lower number of proliferation-competent stem/progenitor cells, ii) a slower proliferation rate, i.e. a prolonged cell cycle, or iii) a shift of stem cells from the proliferative status to quiescence. Our present findings strongly support the latter hypothesis, although a participation of the two other mechanisms cannot be excluded at this time point. It is now crucial to investigate if TGF-beta remodelling of the stem cell niche and induction of stem cell quiescence during the course of neuropathologies is a widespread phenomenon or restricted to a subtype of conditions, such as HD. Under physiological conditions, it is hypothesized that stem cells limit their mitotic activity and remain mostly in quiescence (G0) until a self-renewing cycle is required to maintain a steady state pool and to prevent stem cell pool depletion. While on one hand acute CNS lesions, such as stroke and quinolinic acid- or 6-hydroxydopamine-induced striatal lesions, apparently provide a stimulus to foster stem and progenitor cell proliferation, probably in an attempt to compensate for the neuronal loss\textsuperscript{146, 171, 175} slow progressive neurodegeneration on the other side often compromise
progenitors in their proliferative activity. Whether this inhibitory response serves a specific function or is a negative side-effect deriving from a disease-associated microenvironmental change, such as inflammatory responses, remains to be deciphered. But enhanced neural stem cell quiescence might well serve as a mechanism to maintain or to preserve the stem cell pool. Indeed, the potential to generate neurospheres from the R6/2 mouse is higher compared to WT animals\textsuperscript{94}, and neural progenitors derived from HD mice demonstrate increased neuronal differentiation\textsuperscript{176}.

**TGF-beta1 signaling as a potential mechanism triggering stem cell quiescence in neurodgeneration**

In the frame of several pathological conditions, acute or degenerative, inflammatory processes get activated and a plethora of anti- and pro-inflammatory cytokines, chemokines, neurotransmitters and reactive oxygen species are being released\textsuperscript{177}. The individual pattern of inflammatory cells and cytokines present in the neural stem cell niche during a pathological state might modulate the molecular and cellular context of TGF-beta action and thereby might create apparently diverging TGF-beta-associated effects\textsuperscript{178}. A well known example for this cellular context-dependent Janus-head-like activity of TGF-beta is the fact that TGF-beta acts for most cells as an anti-mitotic cytokine, while it promotes proliferation of tumour cells on the other side\textsuperscript{58}. This context-dependency might explain also conflicting data reporting enhanced cell proliferation in the subventricular zone in HD patients\textsuperscript{147} and diminished progenitor proliferation in the hippocampal neurogenic niche in HD animal models\textsuperscript{98, 149}. We moreover demonstrated in this study that differences in
cellular response could even be documented between the putative stem cell and the neuronal progenitors.

Although the pathways induced by TGF-beta leading to neural stem cell quiescence requires further molecular investigation, mechanisms are likely to be similar to those previously described in other systems, in particular in the hematopoietic system. Observation in the latter system suggested that Pbx1 and Pbx1-dependent genes as well as FoxO3 could be effectors of the TGF-beta-induced quiescence\textsuperscript{179, 180}. FoxO3 is a central stem cell maintenance factor integrating a plethora of signaling cascades including the IL-2R/STAT pathway, the TGF-beta/Smad pathway, the PI3K/Akt/mTOR cascade, and Notch signal and is therefore likely to be involved in the signaling leading to neural stem cell quiescence\textsuperscript{181}.

**What is the impact of the huntingtin transgene expression in neural stem cell biology in tgHD rats?**

In addition to the microenvironment-induced effects on neural stem cell proliferation, the expression of the huntingtin transgene in our animal models might exert cell-intrinsic activities contributing to the cell cycle exit and quiescence. A prerequisite for this however is that the huntingtin transgene gets expressed in neural stem cells. Along the course of the present study, we performed immunodetection for endogeneous and/or transgenic huntingtin using the IC2 and EM48 antibodies in the tgHD rats. However, we could not detect any immunoreactivity in the hippocampal neural stem cell niche (data not shown).

Previously, an increasing expression of the huntingtin protein between 9 and 15 months of age, occurring specifically in mature neurons was reported\textsuperscript{132}. Also, our
analysis of R6/2 mice revealed that mutant huntingtin protein was only present in mature hippocampal granule cells, but not in neuronal precursors or neural stem cells\textsuperscript{149}. Interference with the neuronal maturation process by the mutant and aggregated huntingtin could thus explain the diminished survival of newly generated neurons. Although it cannot be excluded that the presence of minute amounts of huntingtin protein negatively affects neural stem cells proliferation, the main mechanism by which HD reduces neurogenesis appears to proceed via a remodelling of the stem cell niche microenvironment.

In conclusion, the present study demonstrates that neurogenesis in tgHD rat hippocampus is impaired via mechanisms acting at different levels along the generation of new mature neurons. Our evidence supports the hypothesis that defects in progenitor proliferation and cell cycle exit are orchestrated by TGF-beta signaling in the local microenvironment.
6. Summary

In the adult brain, new neurons are continuously produced in the subgranular zone (SGZ) of the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles. This process includes neural stem and progenitor cell maintenance, proliferation, neuronal differentiation, integration and survival. These neural stem cell niches are composed and regulated by its cellular, extracellular matrix and cytokine profile. However, the sequence of cellular events and exact molecular mechanisms that control neurogenesis in normal and neurodegenerative brain are mostly unknown. Transforming growth factor-(TGF) beta1 signaling has been implicated in regulating the stem cell pool during midbrain development, where it controls stem cell proliferation. In the adult brain, it has been demonstrated that experimentally induced levels of TGF-beta1 blocks neural stem and progenitor cell proliferation. Interestingly, adult neurogenesis is inhibited in neurodegenerative disorders such as Huntington’s disease (HD) but the progression of cellular events and molecular mechanisms that manipulate neurogenesis in the HD brain are poorly understood. Surprisingly, the expression of TGF-beta1 and TGF-beta signaling components are elevated in the degenerating HD brain. Therefore, this study investigated 1) the TGF-beta1 signaling in the adult neural stem cell niche under physiological and healthy conditions, 2) the regulation of hippocampal neurogenesis in the brains of a TGF-beta1 inducible transgenic animal model (TGF-beta-on mice), 3) the regulation of neurogenesis in transgenic HD models (tgHD rats and R6/2 mice) at different clinical phases and 4) evaluated a possible correlation between the observed neurogenic modulations and alterations in TGF-beta signaling by performing a comprehensive histological study of TGF-beta1 signaling components in quiescent and proliferating neural progenitors and their progeny.
Results revealed that TGF-beta1 signaling is virtually absent in proliferating stem cells but progressively active in post-mitotic neurons in the hippocampal stem cell niche of the healthy adult brain. An experimentally induced TGF-beta1 level in the hippocampus of TGF-beta1-on mice activates phosphorylation of the downstream signaling component Smad2 in stem cells followed by reduced proliferation of stem and progenitor cells. Moreover, the induced level of TGF-beta1 promoted survival of newly born neurons in the hippocampus of TGF-beta1-on mice. In the tgHD brains, we encountered a disease-associated progressive decline in hippocampal progenitor proliferation accompanied by an expansion of the pool of BrdU-label-retaining Sox2 positive quiescent stem cells. This has been associated with accumulation of pSmad2 in Sox2/GFAP expressing stem cells in the hippocampus of tgHD animals. These results indicate that alterations in neurogenesis in tgHD animals occur in successive phases that are associated with increasing TGF-beta1 signaling. Thus, TGF-beta1 signaling appears to be a crucial modulator of neurogenesis in healthy and HD brains.
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1) *Brain Research* 2007
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Zacharias Kohl*, Mahesh Kandasamy*, Beate Winner, Robert Aigner, Claudia Gross, Sebastien Couillard-Despres, Ulrich Bogdahn, Ludwig Aigner and Jürgen Winkler
(*equal contribution)

2) *Journal of Neurochemistry* 2008
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(All authors contributed equally)

8) **Journal of Neuropathology and Experimental Neurology 2010**
Stem Cell Quiescence in the Hippocampal Neurogenic Niche Is Associated With Elevated Transforming Growth Factor-β Signaling in an Animal Model of Huntington Disease

9) **BMC Neuroscience 2010**
Impaired adult olfactory bulb neurogenesis in the R6/2 mouse model of Huntington’s disease
Zacharias Kohl, Martin Regensburger, Robert Aigner, **Mahesh Kandasamy**, Beate Winner, Ludwig Aigner, Jürgen Winkler

10) **Under submission**
TGF-beta signaling in the adult neural stem cell niche: a decision on stem cell quiescence and neuronal differentiation.
**Mahesh Kandasamy***, Bernadette Lehner*, Olaf Strauss, Sabrina Kraus, Chichung D. Lie, Francisco J. Rivera, Jürgen Winkler, Ulrich Bogdahn, Uwe Überham, Sebastien Couillard-Despres, and Ludwig Aigner

11) **Under Preparation**
Neural precursor’s migration from the SVZ to the striatum in the adult brain of a transgenic rat model of Huntington’s disease
**Mahesh Kandasamy**, Michael Rosskopf, Sebastien Couillard-Despres, Ulrich Bogdahn, Jürgen Winkler, Stephan von Hörsten and Ludwig Aigner
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