Regulation of adult neurogenesis in Huntington's disease: The role of TGF-beta1 signaling in the neurogenic niche

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vorgelegt von

Mahesh Kandasamy

aus

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Die Arbeit wurde angeleitet von: Prof. Dr. Inga Neumann und Prof. Dr. Ludwig Aigner

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Table of Contents

1. Introduction 1
1.1. Adult neurogenesis 1
1.2. History of adult neurogenesis 2
1.3. Stem cells in the adult brain 3
1.4. Hippocampal neurogenesis 4
1.5. Neurogenesis in the SVZ-RMS-OB system 5
1.6. Regulation of adult neurogenesis 7
1.7. Regulation of neurogenesis by signaling molecules 8
1.8. Transforming growth factors 9
1.8.1. The TGF-beta signaling pathway 10
1.8.2. TGF-beta expression in the normal and pathological brain 11
1.8.3. TGF-beta signal transduction in the brain 12
1.8.4. Roles of TGF-beta in the brain 13
1.8.5. Regulation of adult neurogenesis by TGF-beta1 13
1.8.6. Enhanced TGF-beta 1 level and impaired neurogenesis in neurodegenerative disorders 14
1.9. Huntington's disease 15
1.9.1. Clinical aspects of Huntington's disease 15
1.9.2. History of Huntington's disease 16
1.9.3. Epidemiology of Huntington's disease 17
1.9.4. Localization and function of the physiological Huntingtin protein 18
1.9.5. Expanded CAG repeats in the huntingtin gene 19
1.9.6. Neuropathological hallmarks of HD 20
1.9.7. Experimental models of Huntington's disease 21
1.9.7.1. Acute models for Huntington's disease 21
1.9.7.2. Transgenic models of Huntington's disease 21
1.9.8. Neurogenesis in Huntington's disease 22

2. Aim of the study 23

3. Material and Methods 24

3.1. Materials 24
3.1.1. Expendable materials 24
3.1.2. Chemicals for in vivo immunological procedure 25
3.1.3. Chemicals for Western blot 26
3.1.4. Cell culture medias 26

3.1.5. Other reagents for cell culture 27
3.1.6. Buffer, solutions and stock solutions 28
3.1.7. Primary antibodies 31
3.1.8. Secondary antibodies 32
3.1.7. Devices 32
3.1.8. Software 33

3.2. Methods 34
3.2.1. Animals 34
3.2.2. Intracerebroventricular infusions of TGF-beta1 34
3.2.3. BrdU labelling 35
3.2.4. Tissue processing and immunohistochemistry 35
3.2.5. Counting procedures 37
3.2.6. Western Blotting 39
3.2.7. Neural stem and progenitor cells culture 40
3.2.8. Cell cycle analysis 41
3.2.9. Immunocytochemistry 41
3.3. Statistics 42

4. Results 44
4.1. TGF-beta1 signaling components are expressed throughout the adult rat brain 44
4.2. Table 1-Semiquantitative measurement of immunoreactivity of TGF-betaRII, TGF-betaRI and pSmad2 in the adult rat brain 49
4.3. pSmad2 is predominantly present in postmitotic cells in the hippocampus of the adult brain. 51
4.4. Induced over-expression of TGF-beta1 in the hippocampus reduces cell proliferation but promotes neuronal differentiation and survival 54
4.5. Elevated levels of TGF-beta1 provokes expression of pSmad2 in neural stem and progenitor cells 57
4.6. Regulation of hippocampal neurogenesis in tgHD rats 61
4.7. Hippocampal cell proliferation in tgHD rats gets progressively impaired between 8 and 12 months of age 61
4.8. Impaired survival of newly generated cells and reduced neuronal density was mediated by reduced CREB signaling in tgHD rat hippocampus. 61
4.9. Increased quiescence of newly generated cells in tgHD dentate gyrus 65
4.10. Neuroblast proliferation compensates stem cell quiescence in tgHD dentate gyrus 67
4.11. Reduced cell proliferation in tgHD animals correlates with increased TGF-beta1 signaling in hippocampal stem cells 70

5. Discussion **77**

7. Bibliography **88**

Acknowledgements **118**

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

G-CSF	Granulocyte colony-stimulating factor
GDF	Growth differentiation factors
GFAP	Glial Fibrillary Acidic Protein
GS-Domain	Glycin-Serine domain
IGF-1	Insulin-like Growth Factor 1
h	Hour
HC	Hippocampus
HD	Huntington’s disease
Hes	Hairy and enhancer of split
HGF	Hepatocyte growth factor
HIP1	Huntingtin Interacting Protein1
HAP1	Huntingtin-associated protein 1
HPA	Hypothalamic-pituitary-adrenal axis
HRP	Horseradish peroxidase
HSCs	Hematopoietic Stem Cells
HTT	Huntingtin Gene
IT15	Interesting Transcript 15
LAP	Latency Associated Protein
LTBP	Latent TGF-beta-binding proteins
Map2ab	Microtubule-associated Protein 2 Isoform a and b
MBP	Myelin Basic Protein
MSC	Mesenchymal stem cells
min	Minute
MH	Mad Homology
NB	Neurobasal
NGF	Nerve Growth Factor
NI	Neuronal Inclusion
NPCs	Neural Progenitor Cells
NSCs	Neural Stem Cells
NeuN	Neuronal Nuclei
NMDA	N-methyl-D-aspartic acid
OB	Olfactory Bulb
Olig 1	Oligodendrocyte transcription factor1
Olig 2	Oligodendrocyte transcription factor2
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen

pCREB	Phospho cAMP response element-binding
PD	Parkinson's Disease
PFA	Paraformaldehyde
PMFS	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
PRL	Prolactin
pSmad	phospho mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA
RA	Retinoic Acid
RMS	Rostra Migratory Stream
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SEL	Subependymal layer
SF	Scatter Factor
SGZ	Subgranular Zone
Shh	Sonic Hedgehog
SMI94	Antibody against Myelin Basic Protein
Sox2	SRY (sex determining region Y)-box 2
SSC	Saline Sodium Citrate
Smad	mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA
SVZ	Subventricular Zone
Str	Striatum
Stat3	Signal transducer and activator of transcription 3
TBS	Tris Buffered Saline
TgHD rats	Transgenic HD rats
TGF-beta1	Trasnforming growth factor beta1
TGF-beta2	Trasnforming growth factor beta2
TGF-beta3	Trasnforming growth factor beta3
TGF-bR1	Type 1 receptor Trasnforming growth factor beta
TGF-bR2	Type 2 receptor Trasnforming growth factor beta
TGF-bR3	Type 3 receptor Trasnforming growth factor beta
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
WB	Western blot

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).

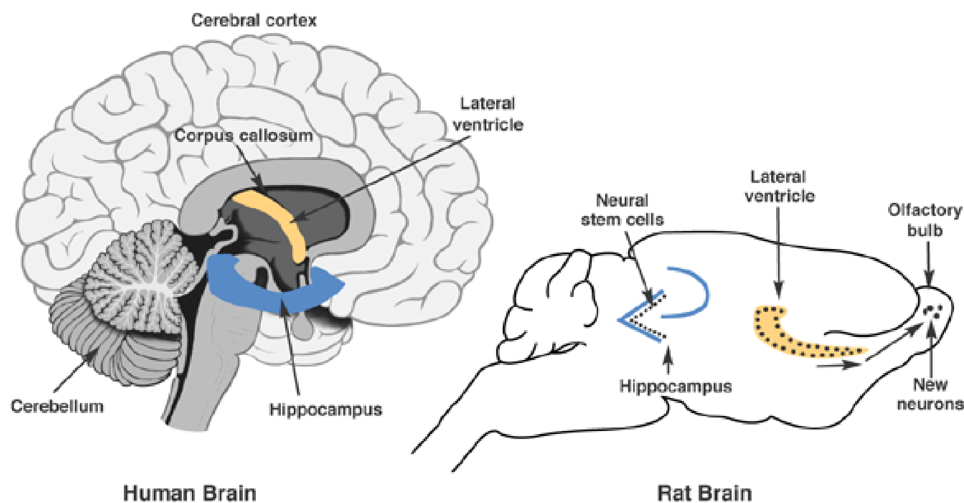

Fig.1.1. Neurogenic niches in the adult human and in the rat brain.
The dentate gyrus of the hippocampus and the subventricular zone are known to produce new neurons in the adult brain. From the subventricular zone of the lateral ventricle cells migrate via the rostral migratory stream to the olfactory 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 [6]. Although there were some occasional reports on mitotic cells in the brain of adult mammals[7] 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 (H^3) thymidine and autoradiography to suggest the production of new neurons in the hippocampus[8]. Later on, it was demonstrated through autoradiography and electron microscopy that the newborn neurons in the hippocampus were structurally integrated [5, 9]. Many advanced techniques emerged in the 1990s. Instead of H^3 thymidine, bromodeoxyuridine (BrdU) and retroviral labelling were used to monitor the newly dividing cells[10-15].

BrdU can be easily detected with immunohistochemical methods and visualized with bright-field and fluorescence microscopes [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 [10, 18, 19]. With the help of these strategies, adult neurogenesis has been demonstrated in numerous mammalian species including humans[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 [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[24] (Fig 1.2). These adult stem cells can be isolated, expanded in culture and also be transplanted [25].

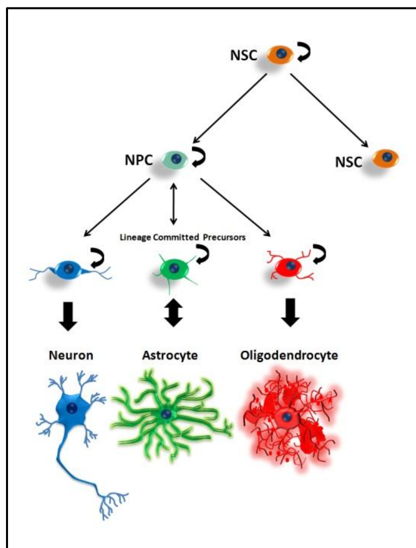

Fig.1.2. Multipotential capacity of neural stem cells. Neural stem cells (NSC) are multipotent and have the capacity to self renew and give rise to lineage-restricted precursor cells, which further differentiate into the three major cell types of the CNS: neurons, astrocytes and oligodendrocytes.

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].

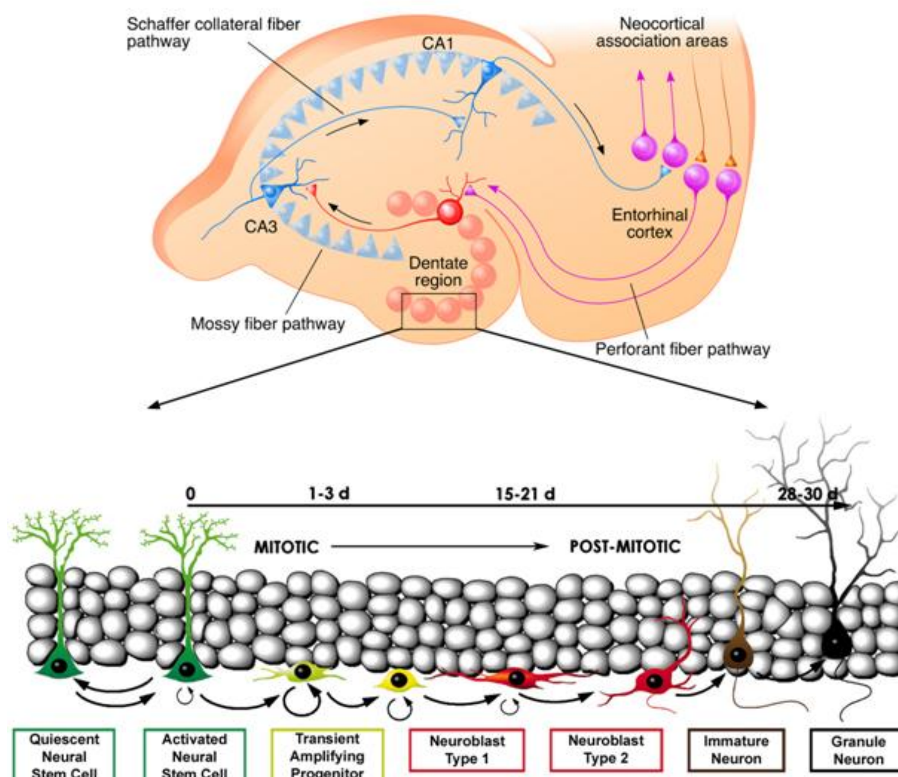

Fig.1.3. Processes of neurogenesis in hippocampal DG
During stage specific event of hippocampal neurogenesis, neural stem cells give rise to mature granular neurons through the production and development of intermediate precursors cells. (Modified from Encinas JM et al., 2006[34])

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)[35]. 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[36, 37]. 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[36, 38, 39] (Fig 1.4).

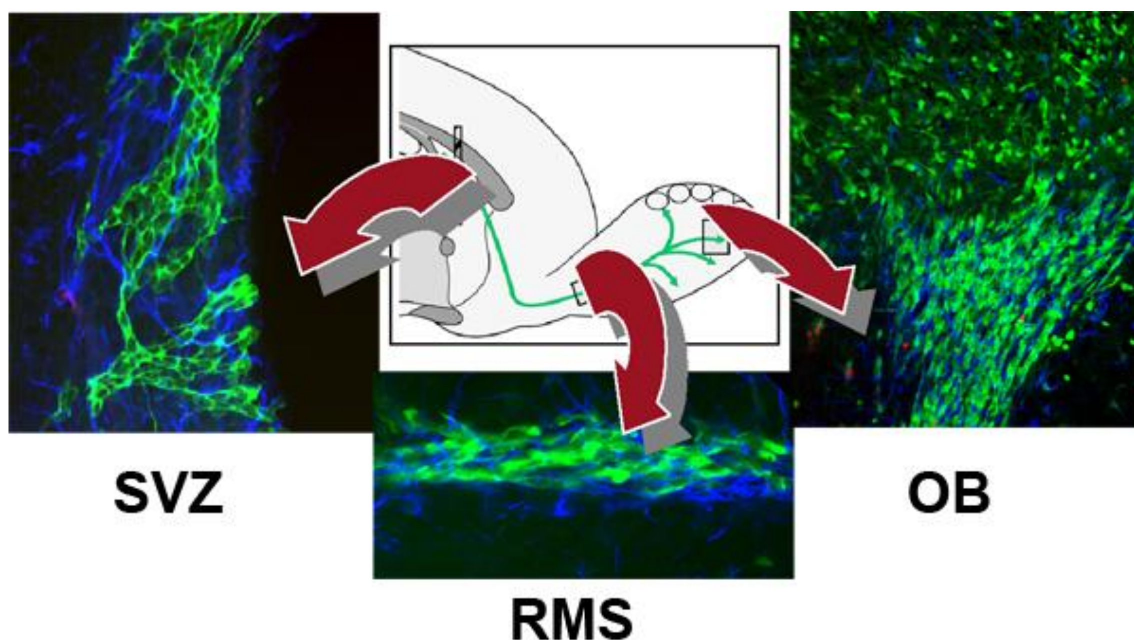

Fig.1.4. Neurogenesis in the SVZ-RMS-OB system
The DCX promoter EGFP transgenic mouse brain represents neurogenesis in the adult olfactory bulb (OB) that is initiated by the persistence of stem cells and progenitors in the subventricular zone (SVZ) of the lateral ventricle. The newly generated cells 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.

1.6. Regulation of adult neurogenesis

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 [23]. 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 [40-42]. 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[16, 43]. The animals that were exposed to an enriched environment and physical exercise showed improved motor skills and better performance in learning tasks [16, 40]. 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[44]. 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[45]. Fluoxetine is an antidepressant drug that has recently been shown to improve neurogenesis during young adulthood[46].

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[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[47-49]. However, this stress induced inhibition of precursor cell proliferation has been shown to be prevented by systemic administration of prolactin (PRL) [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 [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 [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[52]. Moreover, other studies have demonstrated that intracerebral infusion

of IGF increases both cell proliferation and neurogenesis in hypophysectomized rats [53]. Like IGF-1, vascular endothelial growth factor (VEGF) also has a stimulatory effect on neurogenesis[54]. Furthermore, a recent report demonstrated that granulocyte colony-stimulating factor (G-CSF) promotes proliferation of neural progenitors [55].

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[56].

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 pleotrophic cytokines, the TGF-beta molecules[57, 58]. The TGF-beta name was coined in the year 1981 because of its transforming effect on rat kidney and fibroblast cell lines[59-61]. TGF-betas have been implicated in cell proliferation, differentiation, migration, survival, apoptosis, extracellular matrix (ECM) formation, angiogenesis, metastasis, tumorogenesis, inflammation and tissue repair [58]. 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 [57, 62]. 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[63, 64].

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)[65, 66]. 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-betaRI mediated downstream signal by blocking the receptor accessibility to R-Smads (Fig 1.5) [57, 58, 67, 68].

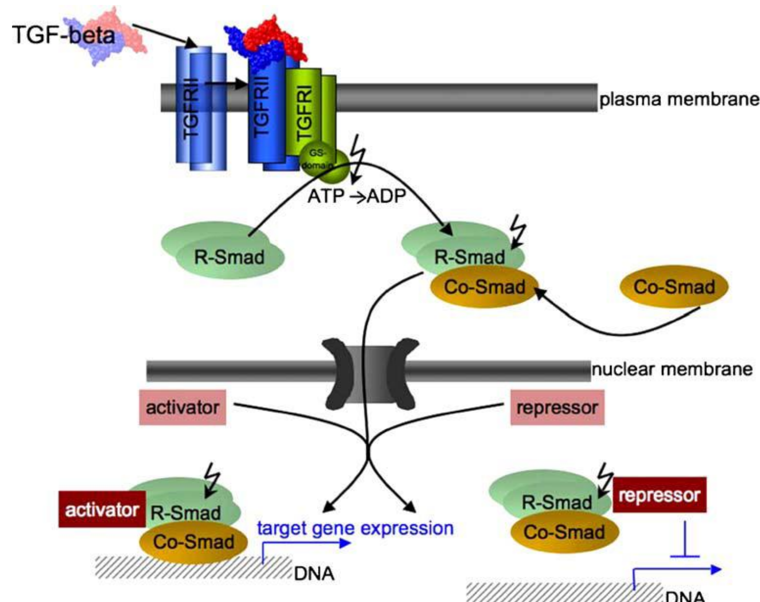

Fig.1.5. Receptor mediated TGF-beta1 signaling
TGF-beta ligand binds to TGF-betaRII that complexes with and activates TGF-betaRI. This induces the downstream Smad-mediated signal transduction (Aigner and Bogdahn, 2008).

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 [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[89]. TGF-beta1 has been shown also to exert a negative effect on the proliferation of microglia [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 neurognic 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 [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[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[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[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[94]. Moreover, TGF-beta1 is an important neuroprotective factor that can prevent neuronal damage from almost all kind of brain damage including neurodegenerative disease[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[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[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 [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[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[101]. This neuronal loss leads to cognitive deficits, impaired motor functions and psychiatric disturbances[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[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[105]. These conditions are known as 'Westphal

variant' of HD and affect 6% of HD patients[106]. Weight loss is one of the features of progressed form of HD due to poor food intake.[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[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 referres 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[109]. However, the disease can encounter at any age and 6% are juvenile forms[110]. The age of onset is closely correlated with the CAG repeat length of the mutant form of the htt gene[111].

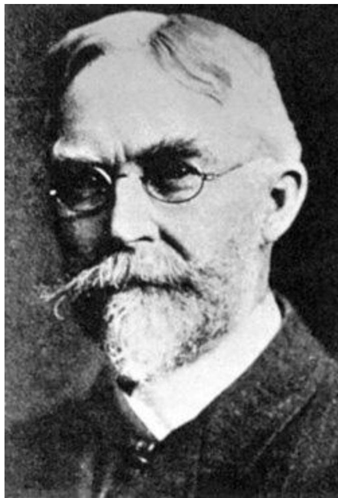

Fig.1.6. A photograph of George Summer Huntington (1850-1916)
Born (1850) in East Hampton, New York, USA –died (1916) in Cairo, New York, USA(Okun, 2003). (http://medinfo.ufl.edu/other/histmed/okun/images/05.jpg)

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[112]. The highest prevalence is in Europe and North America, with 4-8 cases per 100000 people[113]. Venezuela has the highest HD population in the world [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[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[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[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[112, 116]. Htt has very rare homology to other proteins and its functions are poorly understood.

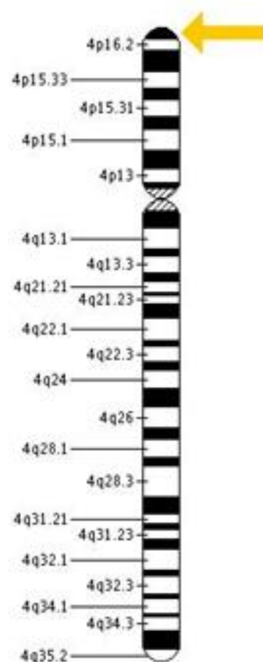

Fig.1.7. Location of the huntingtin gene
Human chromosome 4 with the location of the HD gene indicated at the top of the short arm. (http://ghr.nlm.nih.gov/dynamicImages/chromomap/htt.jpeg)

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[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[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[120]. Furthermore, htt is required for neuronal survival[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 mis folding, 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[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[141]. PolyQ-expressing fruit flies form nuclear inclusions and undergo a progressive neurodegeneration[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[144]. These animals display an early onset of HD pathology, have a shorter life span and die within the first two to four months[144]. Recently, a transgenic rat model of Huntington's

disease(tg HD rat) was developed by von Hörsten and colleagues[135]. 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[132, 135]. Besides, tgHD rats suffer from brain mitochondrial dysfunction and degeneration of medium spiny neurons[71, 145]. 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[132, 135]. 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[144] but it is increased in the SVZ of chemically induced acute models of the disease[146] and HD patients[147]. 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

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

3.1.2 Chemicals for in vivo immunological procedure

Acepromazine	WDT, Garbsen, Germany
Vectastain Elite ABC kit	Vector Laboratories, Burlingame, USA
BrdU	Sigma-Aldrich, Taufkirchen
BSA	Sigma-Aldrich, Taufkirchen
3,3'-Diaminobenzidine (DAB)	Vector Laboratories, Burlingame, USA
Ethylene glycol	AppliChem, Darmstadt
Formamide	Merck, Darmstadt
Glycerol	AppliChem, Darmstadt
HCl	Merck, Darmstadt
H_2O_2	Vector Laboratories, Burlingame, USA
	Merck, Darmstadt
Ketamine	WDT, Garbsen, Germany
Methanol	Merck, Darmstadt
NaCl	Sigma-Aldrich, Taufkirchen
NaOH	Sigma-Aldrich, Taufkirchen
NaH2PO4	Merck, Darmstadt
Na2HPO4	Merck, Darmstadt
Nuclear	Merck, Darmstadt
Neo-Mount	Merck, Darmstadt
Nicl2	Vector Laboratories, Burlingame, USA
Paraformaldehyde (PFA)	Sigma-Aldrich, Taufkirchen
Prolong Antifade kit	Invitrogen Molecular Probes™ Eugene, Oregon, USA
Teleostean gelatin	Sigma-Aldrich, Taufkirchen
Topro3	Molecular Probes, USA
Triton X 100	Sigma-Aldrich, Taufkirchen
Sucrose	Sigma-Aldrich, Taufkirchen
Tissue Tek	Sakura finetek, USA
Tris-Base	Sigma-Aldrich, Taufkirchen
xylazine	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

Trypsin	PAN, Germany

3.1.6 Buffer, solutions and stock solutions

Tris Buffered Saline (TBS)	800 ml dH_2O
	8g NaCl
	3g Trisbase
	HCl adjust to pH 7.4
Borate Buffer (0.1M)	3.08g boric acid
	450 ml H_2O
	5N NaOH to pH 8.5
	Final volume to 500 ml
PBS (0.1M)	500ml 0.2M Phosphate Buffer
	500ml dH_2O
	9g Natriumchloride
Phosphate Buffer (0.2M)	1L dH_2O
	5.52g NaH_2PO_4, water free
	21.9g Na_2HPO_4, water free
	10ml 10xPBS
Cryo Protective solution	250 ml Glycerin
	250 ml Ethylenglycol
	500 ml 0.1M PO_4 buffer.
Fish Skin Gelatin Buffer (FSGB)	0.1M Tris-HCl, pH 7.5
	0.15M NaCl
	1% w/v BSA
	0.2% v/v Fish Skin Gelatin

	0.1% v/v Triton X-100
DAB solution	0.25 mg/ml 3, 3′-diaminobenzidine 0.01% (v/v) H_2O_2, 0.04% (w/v) $NiCl_2$
Donkey serum blocking buffer	0.1 M Tris-Hcl, pH 7.5 0.15 M NaCl 3% donkey serum 0.1% Triton X-100
4% Paraformaldehyde (PFA) For perfusion	250 ml dH20 0.5 ml 10M NaOH 20 g PFA 250 ml 0.2M PO_4 buffer
4% Paraformaldehyde (PFA) For fixation of cells	4% PFA 2.5mM NaOH 0.4mM $CaCl_2$ 50mM Sucrose 0.1M NaH_2PO_4
Protein Isolation Buffer (Fei He et al. 2005 modified)	0.7% IGEPAL 50mM Tris HCl, pH 8.0 0.1mm EDTA pH 8.0 250mM NaCl 10% Glycerol 0.2mm Na_2VO_4 50mM NaF 1mM PMSF 10mM DTT Protease inhibitors
Sample buffer for proteins-WB	1M Tris-HCl, pH 6.8

	1% SDS 30% Glycerol DTT 2% bromophenol blue
Resolving gel buffer -WB	2M Tris-HCl pH 8.0 10% SDS H_2O bidest
Stacking gel buffer -WB	1M Tris-Cl, pH 6.8 10% SDS H_2O bidest
Electrophoresis buffer (1L)-WB	25mM Tris Base (3g) 14,4g Glycin 1% SDS
Blotting Buffer-WB	25mM Tris Base (3,03g/L) 150mM Glycine (11,26g/L)
PPD (100 ml)	0.01% Papain 0.1% Dispase II 0.01% Dnase I 149 mg MgSo4*7H2O in Hank's Balanced Salt Solution w/o Ca2+/Mg2+
Running buffer for WB	3.02g Tris-Base 14.4g glycerin 1.0g SDS dissolve in 0.8l H2O, set at pH 8,3 with 2M HCl and fill up to 1l with H2O
30% Succrose	400ml 0.1M PO4 150g Sucrose

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

List of Antibodies	Marker	Dilution for Histology	Dilution for WB	Source
Rat anti-BrdU	Base analogue	1:500		Oxford Biotechnology, UK.
Mouse anti-PCNA	Proliferation	1:500		Santa Cruz, USA
Rabbit anti-GFAP	Astrocytes	1:100		Dako, Denmark
Guinea pig anti-GFAP	Astrocytes	1:500		Progene, Germany
Mouse anti rat-Nestin	Stem cell	1:500		Pharmingen, USA
Goat anti-Sox2	Stem cell	1:500		Santa Cruz, USA
IgM mouse anti-A_2B_5	Glial progenitor	1:200		Chemicon, USA
Rabbit anti-DCX	Neuroblast	1:500		Chemicon, USA
Goat anti-DCX	Neuroblast	1:2000		Santa Cruz, USA
Mouse anti-MAP2ab	Neuron	1:500		Sigma, Germany
Mouse anti-NeuN	Neuron	1:500		Chemicon, USA
Mouse anti-MBP	Oligodendrocyte	1:500		Sternberger Monoclonals Incorporated, USA
Mouse anti-TGF-bRII	TGFb-signaling	1:50	1:100	Santa Cruz, USA
Rabbit anti-TGF-bRI	TGFb-signaling	1:50	1:1000	Santa Cruz, USA
Rabbit anti-pSmad2(Ser465/467)	TGFb-signaling	1:50	1:1000	Cell Signaling, USA
Mouse anti Smad2	TGFb-signaling		1:1000	Cell Signaling, USA
Rabbit anti-pCREB(Ser133)	CREB-signaling	1:100	1:1000	Cell Signaling, USA
Rabbit anti-CREB	CREB-signaling		1:1000	Cell Signaling, USA
Rabbit anti-Actin	Loading control		1:5000	Sigma, Germany

3.1.7. Secondary antibodies

List of Antibodies	Dilution	Source
Donkey anti-rabbit-HRP	1:10000	Dianova, Germany
Goat anti-mouse HRP	1:5000	Chemicon, USA
Donkey anti-rat- BT	1:500	Jackson Immuno Research,USA
Donkey anti-goat-BT	1:500	Jackson Immuno Research,USA
Donkey anti-rabbit-Alexa 488	1:1000	Molecular Probes, USA
Donkey anti-goat, Alexa 488	1:500	Molecular Probes, USA
Donkey anti-mouse- Alexa 488	1:500	Molecular Probes, USA
Donkey anti-goat, Alexa 568	1:1000	Molecular Probes, USA
Donkey anti-mouse- RHOX	1:1000	Dianova, Germany
Donkey anti-rabbit - Alexa 568	1:1000	Molecular Probes, USA
Donkey anti-mouse- Cy5	1:1000	Jackson Immuno Research,USA
Donkey anti- guinea pig - Cy5	1:500	Jackson Immuno Research,USA
Donkey anti- mouse IgM - Alexa 568	1:500	Molecular Probes, USA

3.1.9. Devices

Device	**Company**
Tubing pump	Ismatek SA-Switzerland
Sliding microtome	Leica, Solms Diagnostic Instruments, USA
Water bath	GFL,Germany
Slide Moat	Boekel Scientific,USA
Orbital Shaker	colonial scientific, USA
Weighing Balances	Sartorius, Gottingen
Stereology	Stereoinvestigator, MicroBrightField, Colchester, USA
Fluorescence Microscope Leica DMR with SPOT Camera	Leica, Wetzlar, Germany
Confocal scanning laser microscope	Leica TCS-NT, Wetzlar, Germany
Inverse Fluorescence microscope Olympus IX 70 with Color View documentation system	Olympus, Hamburg Soft Imaging Systems, Münster

Light microscope Olympus CK 30	Olympus, Hamburg
Photometer Ultrospec 2000	Amersham/Pharmacia Biotech, Freiburg
Centrifuge 5417 R	Eppendorf, Hamburg
Megafuge 1.0 R	Heraeus Instruments GmbH, Germany
Emax Precision Microplate Reader	Molecular Devices, Union City, CA, USA
Incubator HERA Cell	Heraeus, Germany
Hera Safe cell culture hood	Heraeus, Germany

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[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)[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[149] and from TGF-beta infused rats [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[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 Immunohistrochemistry

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_2O_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[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

Santa Cruz Labs, Santa Cruz, USA), mouse anti-NeuN (neuronal nuclei) 1:500 (Chemicon, Temecula, USA), rabbit anti-GFAP (Glial Fibrillary Acidic Protein) 1:1000 (Dako, Denmark), rabbit anti-pSmad2 (Phospho-Smad2 (Ser465/467) 1:50 (Cell Signaling, Denver, USA), goat anti- Sox2 1:500 (Santa Cruz Labs, Santa Cruz, USA). The Secondary antibodies: donkey anti-goat, -mouse, -rabbit or -rat conjugated with Alexa 488 (1:1000, Molecular Probes, Eugene, USA), rhodamine X (Dianova, Hamburg, Germany), Cy5 or biotin 1:500 (Jackson Immuno Research, West Grove, USA).

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.,[151] and Williams & Rakic (1988)[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/mm3. 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[153]. To analyze proliferating units, size of clusters (> 3 cells) in µm2 and number of BrdU cells per clusters (proliferating units) were quantified on a Olympus microscope (Olympus, Hamburg, Germany) using analySIS® 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, Taufkirchen, Germany) the resulting supernatants (20 ug 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 (AmershamPharmacia, Freiburg, Germany).

3.2.7. Neural stem and progenitor cells culture

Adult hippocampal progenitor cells were isolated and cultured as described before[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°C in a humidified incubator with 5% CO_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 5×10^5 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[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 Win MDI 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-A_2B_5 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., A_2B_5), 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 photo-documentation 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 G 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 [65]. 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).

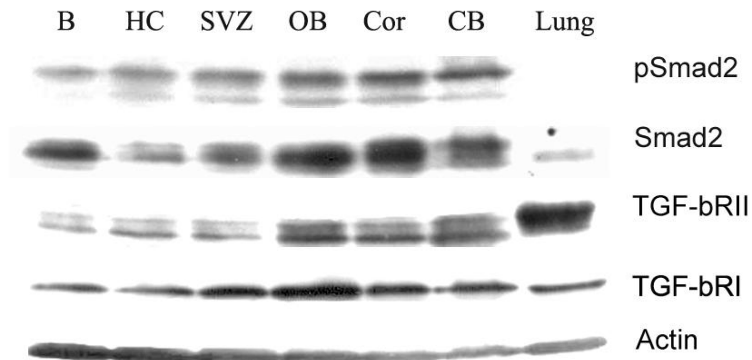

Fig.4.1. Westernblot analysis demonstrating TGF-beta1 signaling components in intact adult brain. Note the expressions of TGF-betaRII, TGF-betaRI and phosphorylated form of Smad2 in different brain regions. B-Brain, HC-Hippocampus, SVZ-Sub ventricular Zone, OB-Olfactory bulb, Cor-Cortex, CB-Cerebellum 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 (Table1 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.

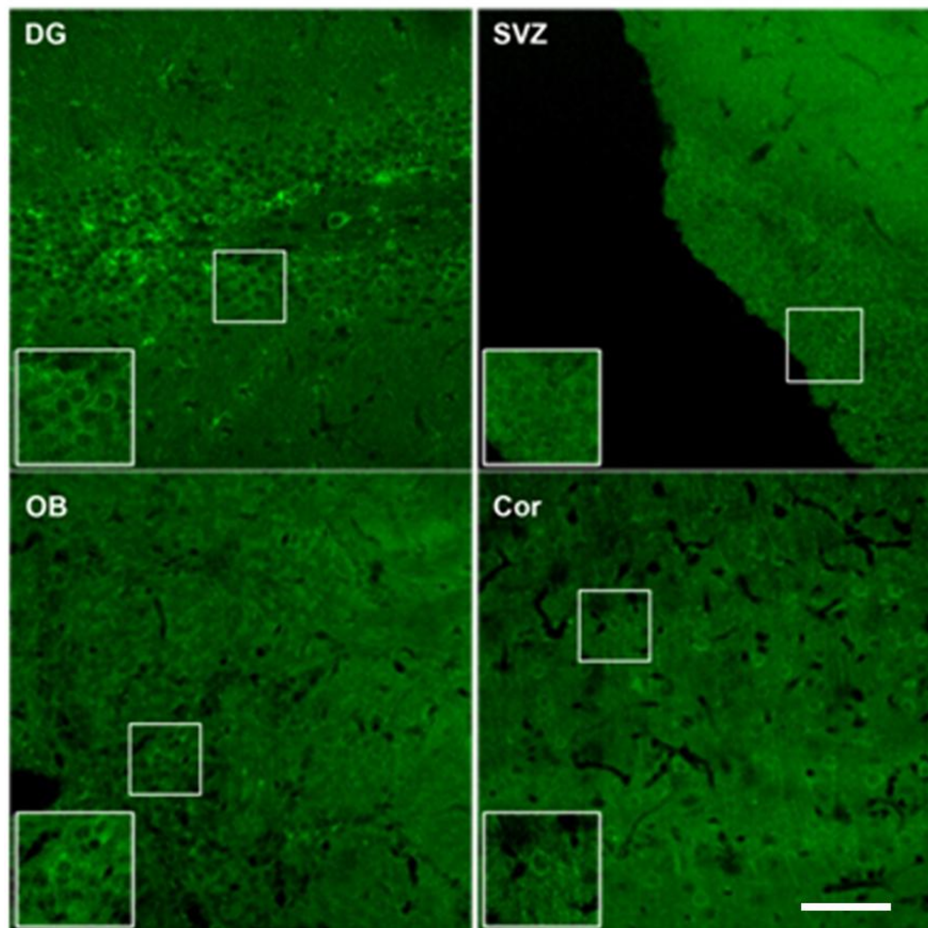

Fig.4.2. Localization of TGF-betaRII (green) immunoreactivity in different areas of the intact adult rat brain. Note the expression of TGF-betaRII in the DG-Dentate Gyrus, in the SVZ-Subventrivular Zone, in the OB-Olfactory bulb and in the Cor-Cortex. scale bar, 50μm. Insets are higher magnifications of selected fields.

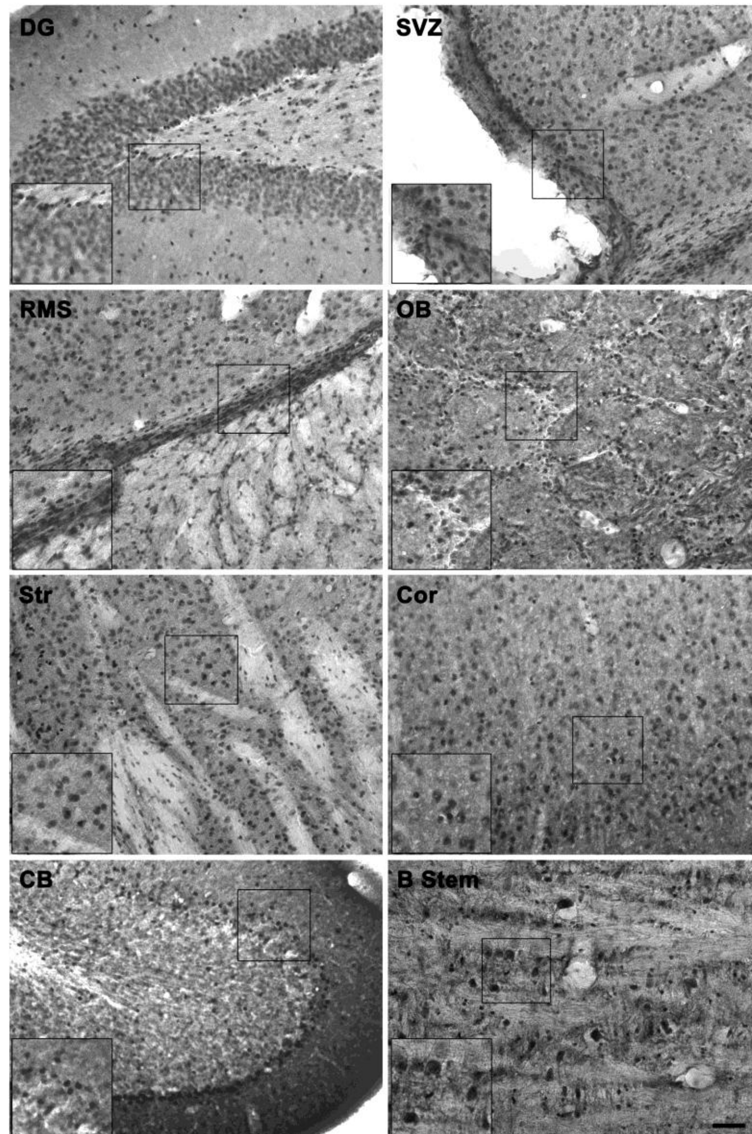

Fig.4.3. Localization of TGF-betaRI immunoreactivity in different areas of intact adult rat brain. Note the widespread expression of TGF-betaRI in the DG-Dentate Gyrus, in the SVZ-Subventrivular Zone, in the OB-Olfactory bulb, in the Str-Striatum, in the Cor-Cortex, in the CB-Cerebellum and in the B Stem-brain stem. scale bar, 100 µm. Insets are higher magnifications of selected fields.

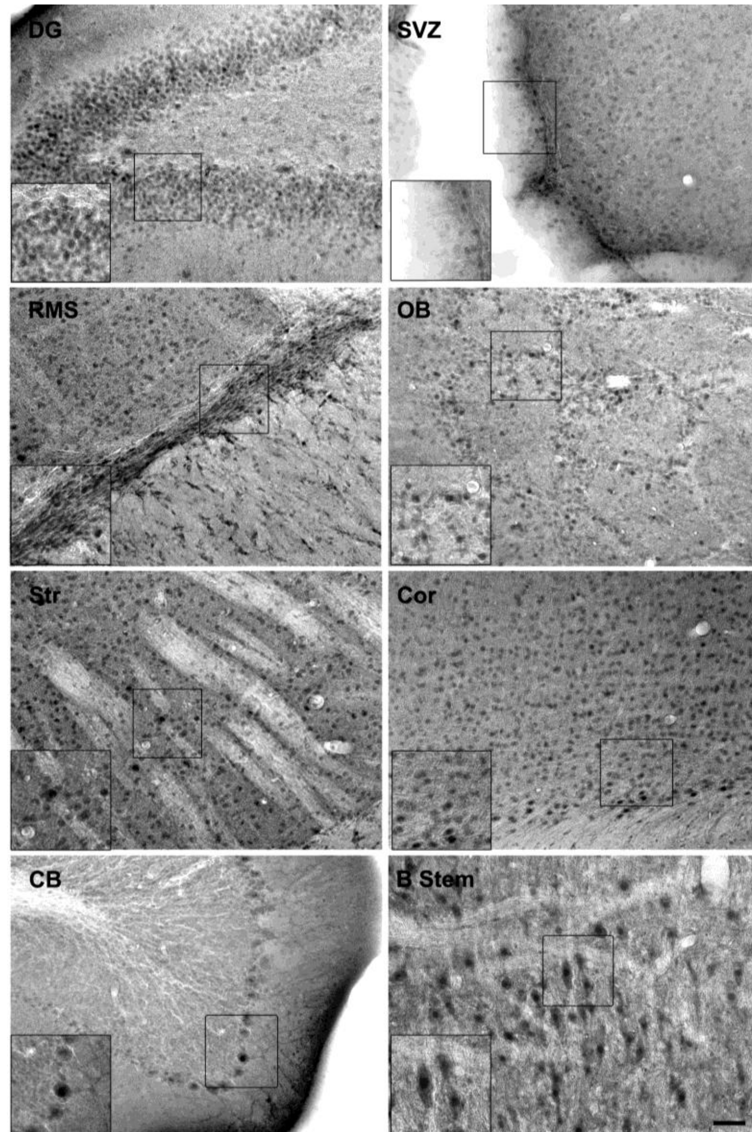

Fig.4.4. Localization of pSmad2 immunoreactivity in different areas of intact adult rat brain. Note the phosphorylated form of pSmad2 expression in the DG-Dentate Gyrus, SVZ-Subventrivular Zone, OB-Olfactory bulb, Str-Striatum, Cor-Cortex, CB-Cerebellum and B Stem-brain stem. However, it is highly limited 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

Brain Area	TGF-betaRII	TGF-betaRI	pSmad2
TELENCEPHALON			
Olfactory system			
Glomerular cell layer	++	++++	++
Granule cell layer	+	++++	++++
Neocortex			
Ventrolateral orbital cortex	++	++++	+++
Frontal cortex	+++	++++	+++
Parietal cortex	+	++++	+++
Occipital cortex	++	++++	++++
Entorhinal cortex	+++	+++	++++
Metacortex			
Cingulate/retrosplenial cortex	+	+++	++++
Hippocampal formation			
Dentate gyrus(GCL)	+	++	+++
Hilus dentate gyrus	<+	+++	+
CA1 region	++	+++	+++
CA2 region	<++	+++	+++
CA3 region	+	+++	+++
Hippocampal fissure	+	++	-
Subiculum	+	++	++
Fimbria of hippocampus	++	++	-
Basal ganglia			
Striatum	++	++++	+++
Globus palidus	+	++++	+
Nucleus accumbens	++	++	+
Amygdala			
Central amygdaloid nucleus	++	+++	+

Medial amygdaloid nucleus	<+	++	+
DIENCEPHALON			
Thalamus	+	+++	+
Hypothalamus	++	++++	+++
MESENCEPHALON			
Substantia nigra pars compacta	+	++	++
Substantia nigra pars reticulate	++	++	+++
Subthalamic nucleus	<+	+++	+
Ventral tegmantal area	<+	+++	+
Red nucleus	+++	+++	++
Superior colliculus	<+	+++	++
Inferior colliculus	<+	+++	++
METENCEPHALON			
Pons	++	++	++
Cerebellum			
Granular cell layer	+	++++	+
Purkinje cell layer	++	+++	++++
Deep cerebellar nuclei	<++	+++	++++
Molecular layer	++	+	+
White matter	++	+	-
Nonneuronal cells			
Choroid plexus	+++	(++++)	+
Ependymal cells	<+	++++	+++
Neurogenic areas			
Subventricular zone	<+	++++	+
Subgranular zone	+	+++	+
Rostral Migratory Stream(RMS)	<+	++++	++

Table.1. Each brain region was evaluated for the relative density of TGFbetaRII, TGFbetaRI 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[150] into immature (short horizontal processes) and mature (perpendicular dendritic arborization into the molecular layer) DCX positive cells[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.

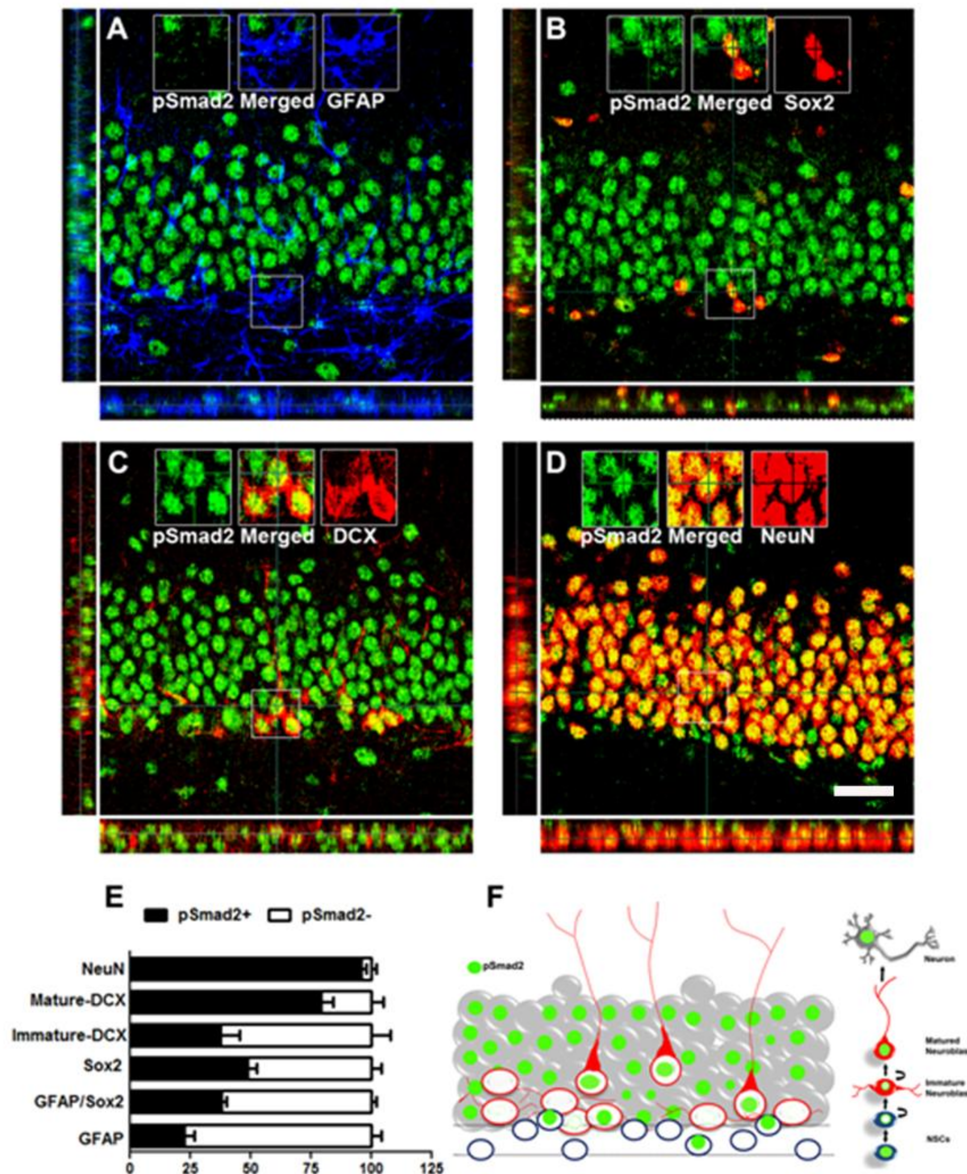

Fig.4.5. Expression of pSmad2 (green) in stem cell niche of the intact adult rat brain.

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 hipocamapal 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.

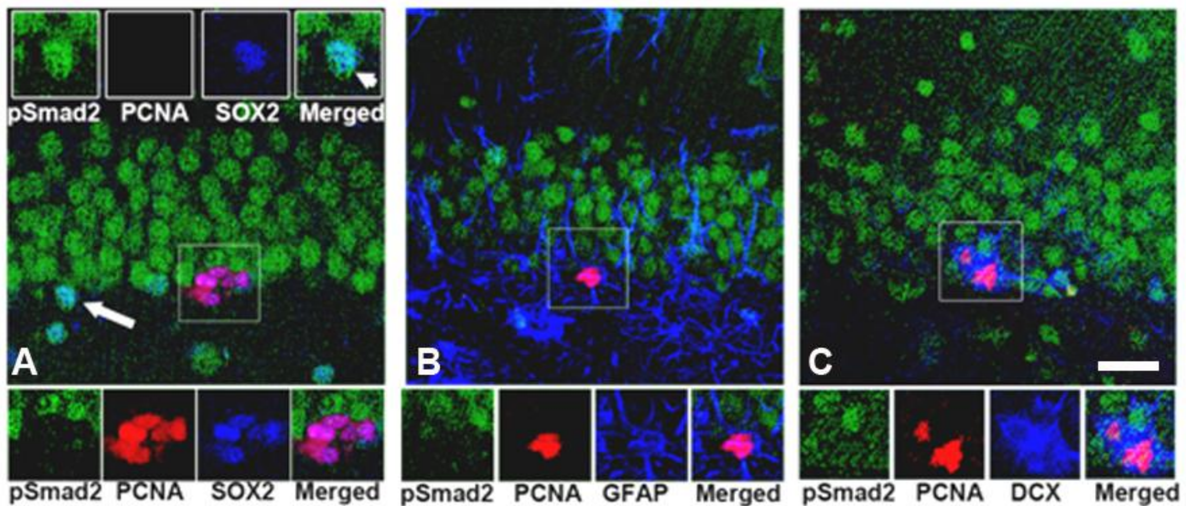

Fig.4.6. TGF-beta1 signaling is absent in proliferating stem and progenitor cells A) Absence of pSmad2(green) in Sox2 (blue)/PCNA (Red) double positive cells in the SGZ of the hippocampus but arrow shows co-localization of pSmad2 (green) in Sox2 (blue) positive /PCNA negative cell(red). B) Absence of 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[82]. 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 [148].

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 immunereactivity 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 surving 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).

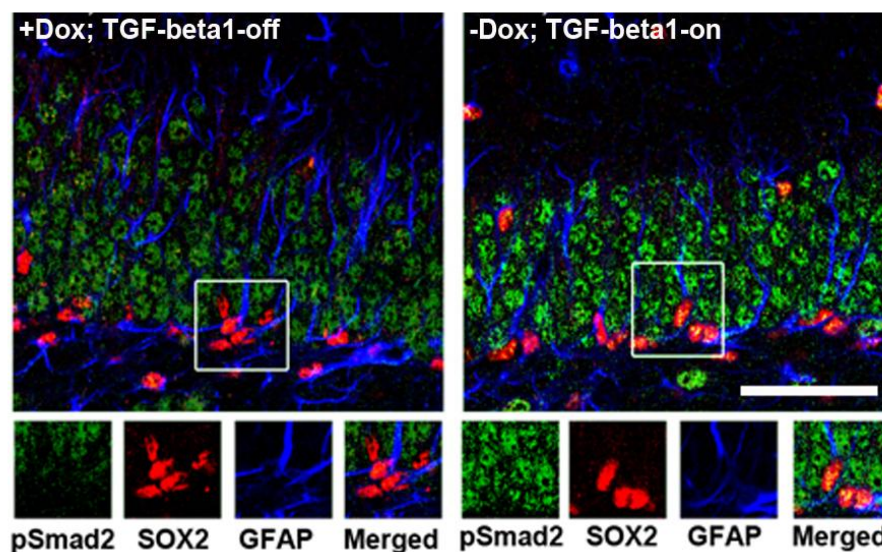

Fig. 4.7a. pSmad2 staining in hippocampal DG of TGF-beta-off and -on mice. Note the prominent immunoreactivity of pSmad2 in the hippocampal DG of TGF-beta on mice. pSmad2 (green) is strongly present in Sox2 (red)/GFAP (blue) double

positive cells specifically in the SGZ of the hippocampus from TGF-beta1-on mice. Scale bar, 100 μm.

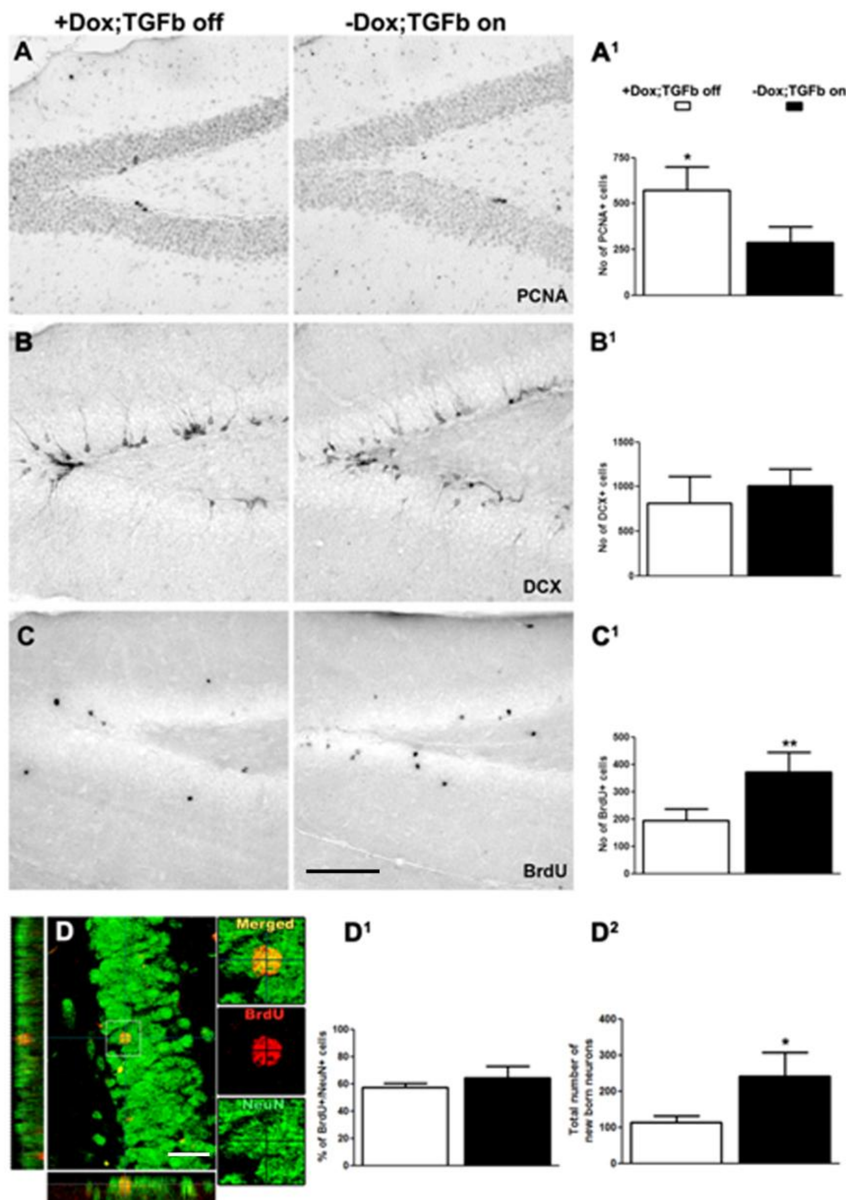

Fig.4.7.b. Reduced proliferation but increased neuronal survival in the hippocampus of TGF-beta-on mice

A-A[1]) Qunatification 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[1]*p<0.05). B-B[1]) Qunatification 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[1]). C-C[1]) Qunatification 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[1]**p<0.01). D-D[2]) Qunatification of the phenotype of BrdU positive cells. Note the increased total number of BrdU/NeuN doublpositive 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 neuro-sphere 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 A_2B_5 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 [82].

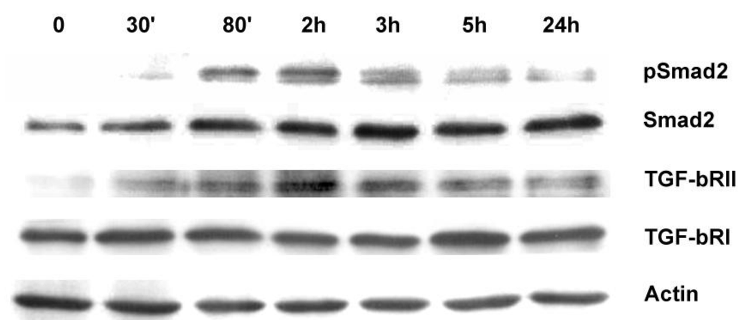

Fig.4.8. Western blot analysis demonstrating the kinetic regulation of TGF-beta1 signaling in stem and progenitor cells in vitro. Note the trace amount of expressions of TGF-beta RII in NSC-NPCs, it is slightly up regulated at 80 mins and 2hr upon TGF-beta1 stimulation while expression of TGF-bRI showed 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.

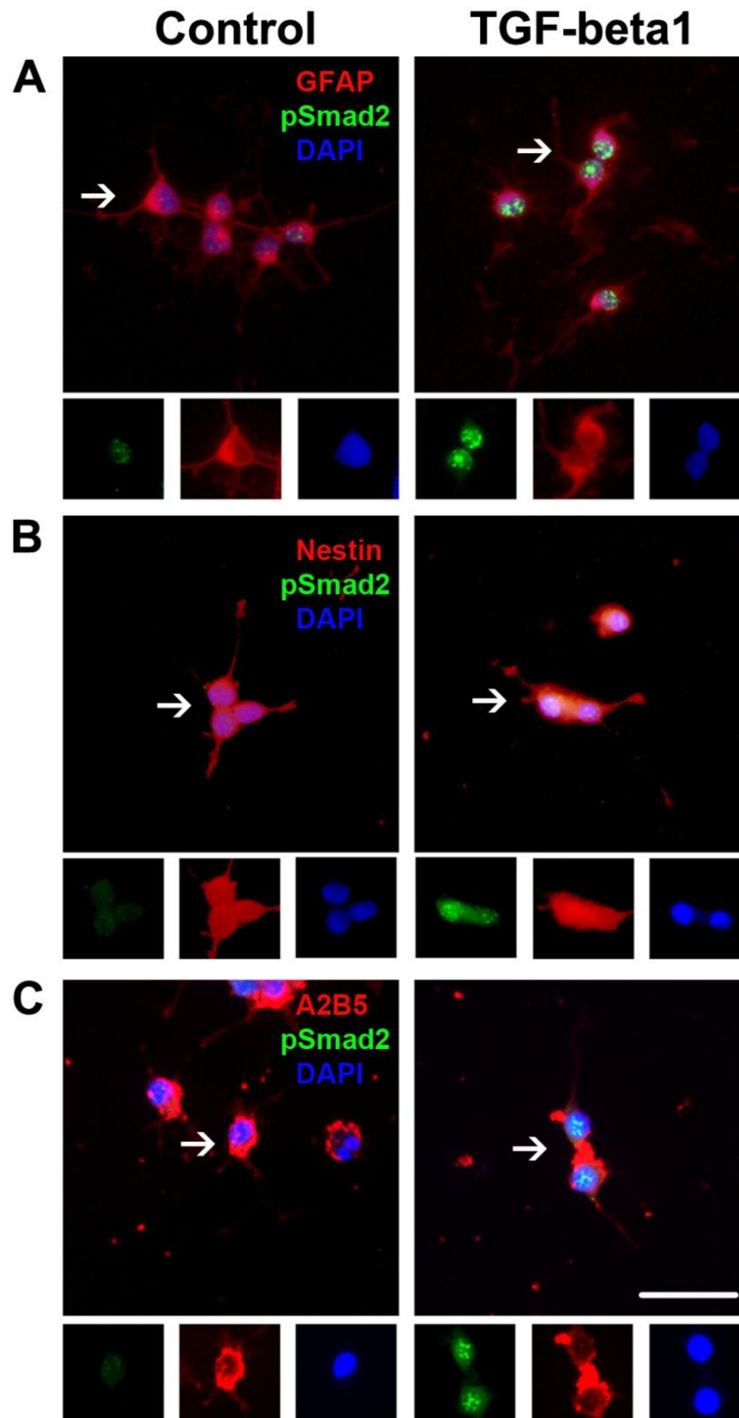

Fig.4.9. Immunostaning of pSmad2 in stem cell and progenitor cells in vitro with or without TGF-beta1 treatment. A) Note the translocation of pSmad2 (green) into nucleus (blue-DAPI) A) of GFAP positive cells(red), B) of Nestin positive cells(red) and C) of A_2B_5 cells (red) in TGF-beta1 treatment, while 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).

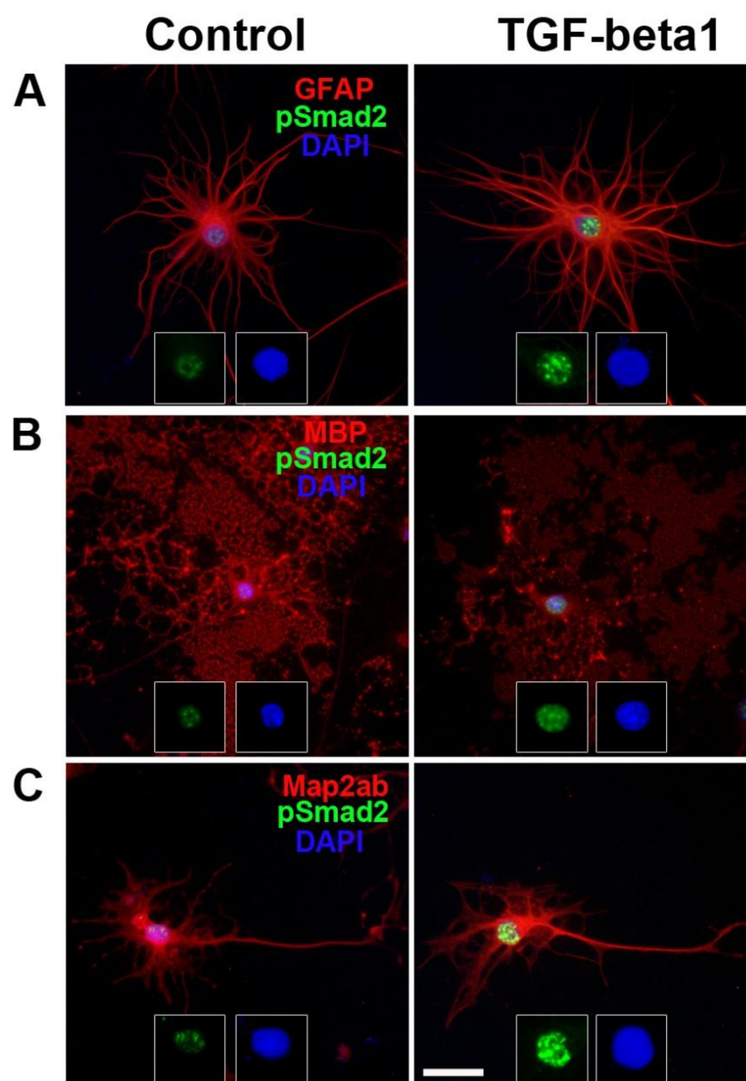

Fig.4.10. Immunostaning of pSmad2 in matured cell populations of the CNS at invitro status with or without TGF-beta1 treatment. Note the translocation of pSmad2 (green) into nucleus (blue-DAPI) A) of GFAP positive astrocytes (red), B) of MBP positive oligodendrocytes (red) and C) of MAP2ab positive neurons (red) in TGF-beta1 treatment, while control condition show a basal amount pSmad2 expression. Note the down regulation of MBP expression in oligodendroctes 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[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[132]. Given that working memory is tightly associated with hippocampal function and requires persisting neurogenesis [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).

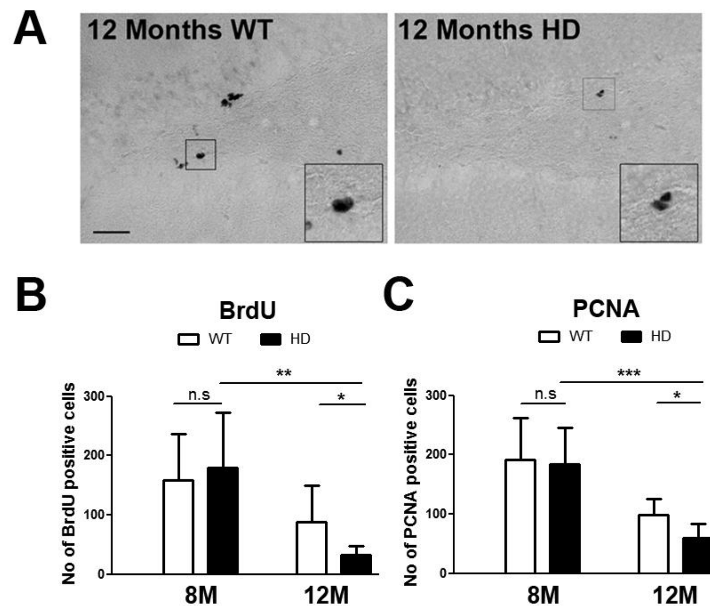

Fig.4.11. Impaired hippocampal proliferation in 12 month-old tgHD rats.
BrdU labelling of 12 month-old WT and tgHD hippocampus (A). Insets are higher magnifications of the selected fields. Scale bar, 100 μm. Quantitative analysis of number of BrdU positive cells in 8 months (B) and 12 months (C) rats 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

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 [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[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/mm3) 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.

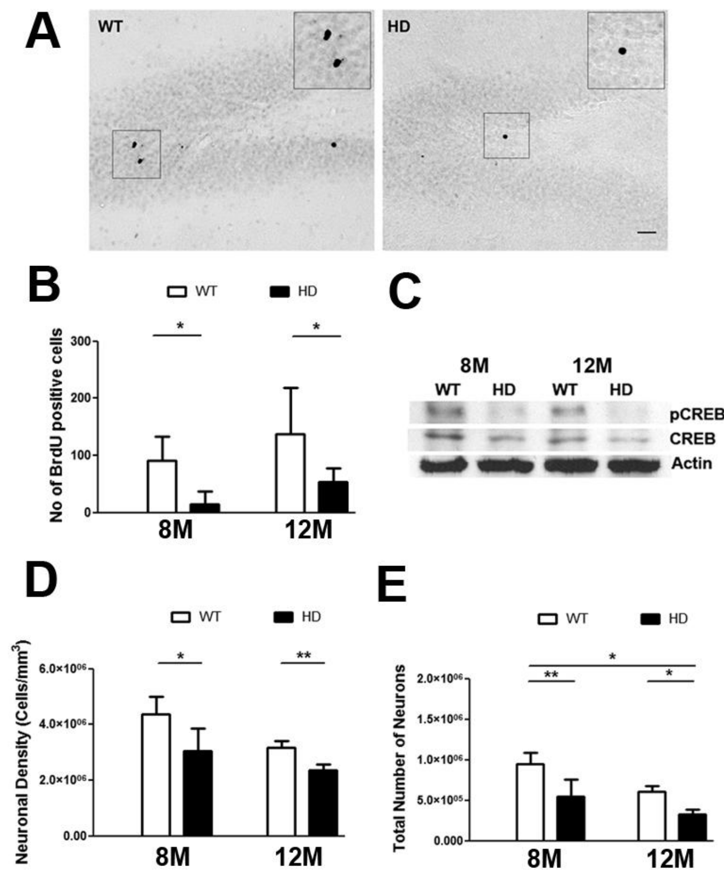

Fig.4.12. Reduced cell survival in tgHD rats is associated with diminished CREB signaling.

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 lableld 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.

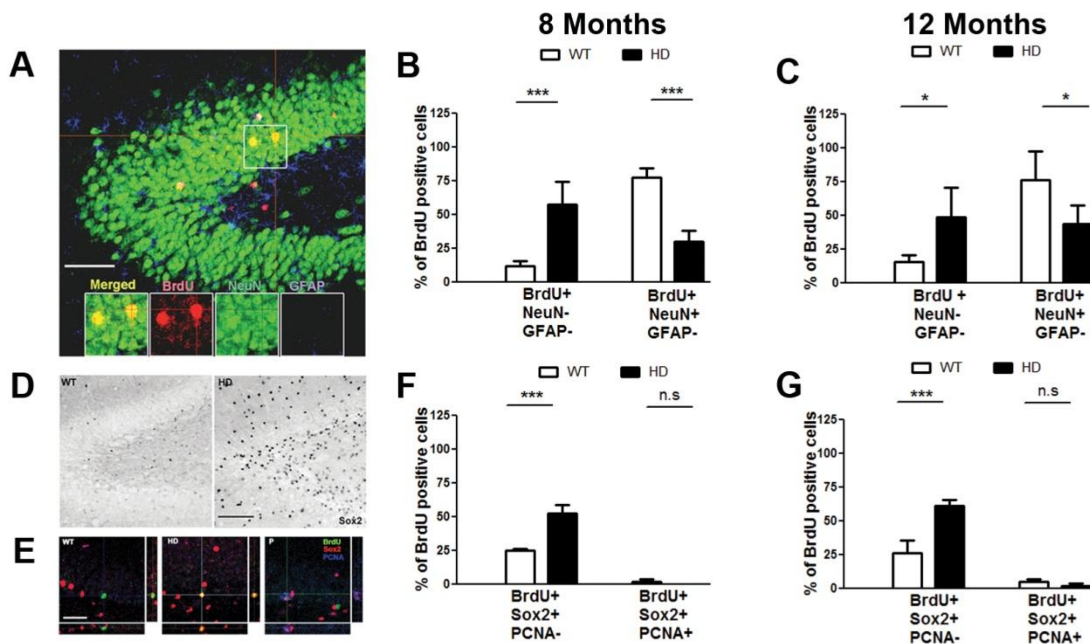

Fig.4.13. Deficiency in neuronal differentiation and augmentation of quiescence in stem cells in tgHD rats.

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.

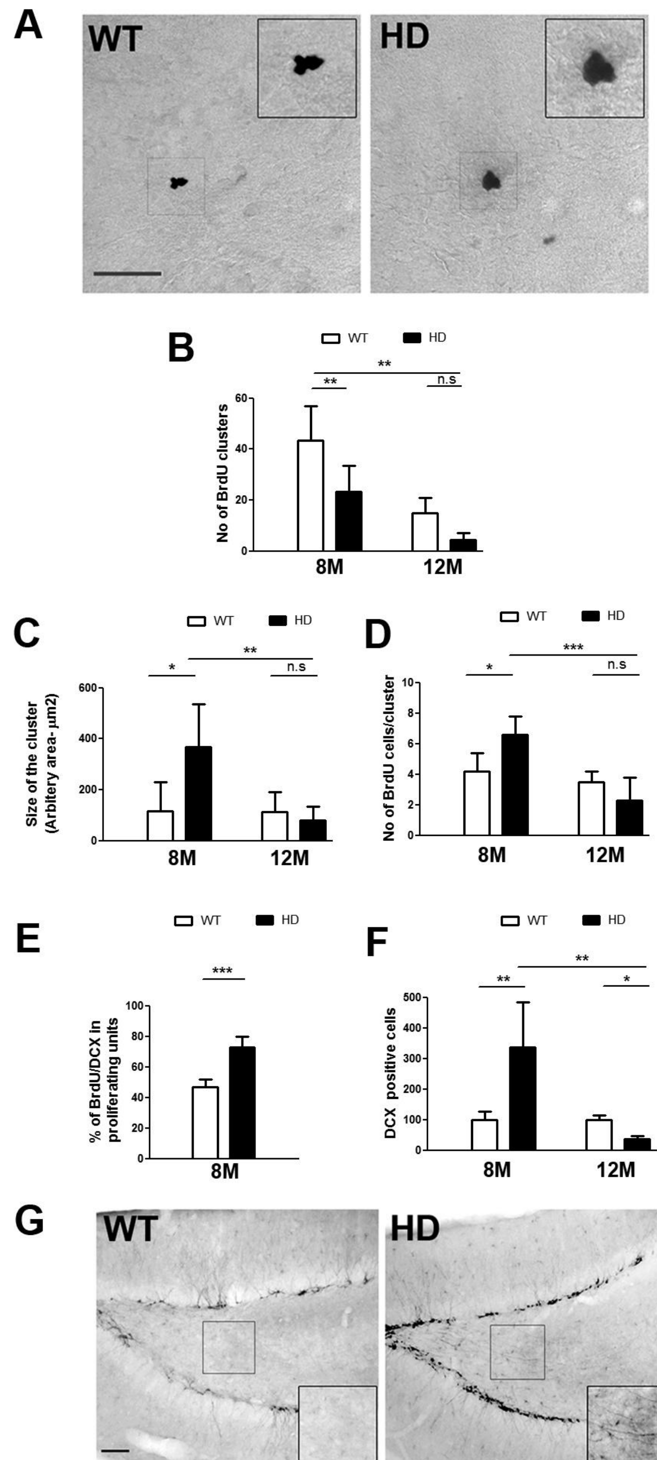

Fig. 4.14. Neuroblast proliferation in 8 month old tgHD hippocampus.
A) Clusters of BrdU positive cells indicating proliferative units in 8 month-old WT and tgHD DG 24 hrs after BrdU injection; scale bar, 50µm. Quantitative analysis of (B) number of BrdU clusters, (C) size of BrdU clusters, (D) number 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 tgHD in 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 [58, 82, 91]. 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[71]. 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[57], 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).

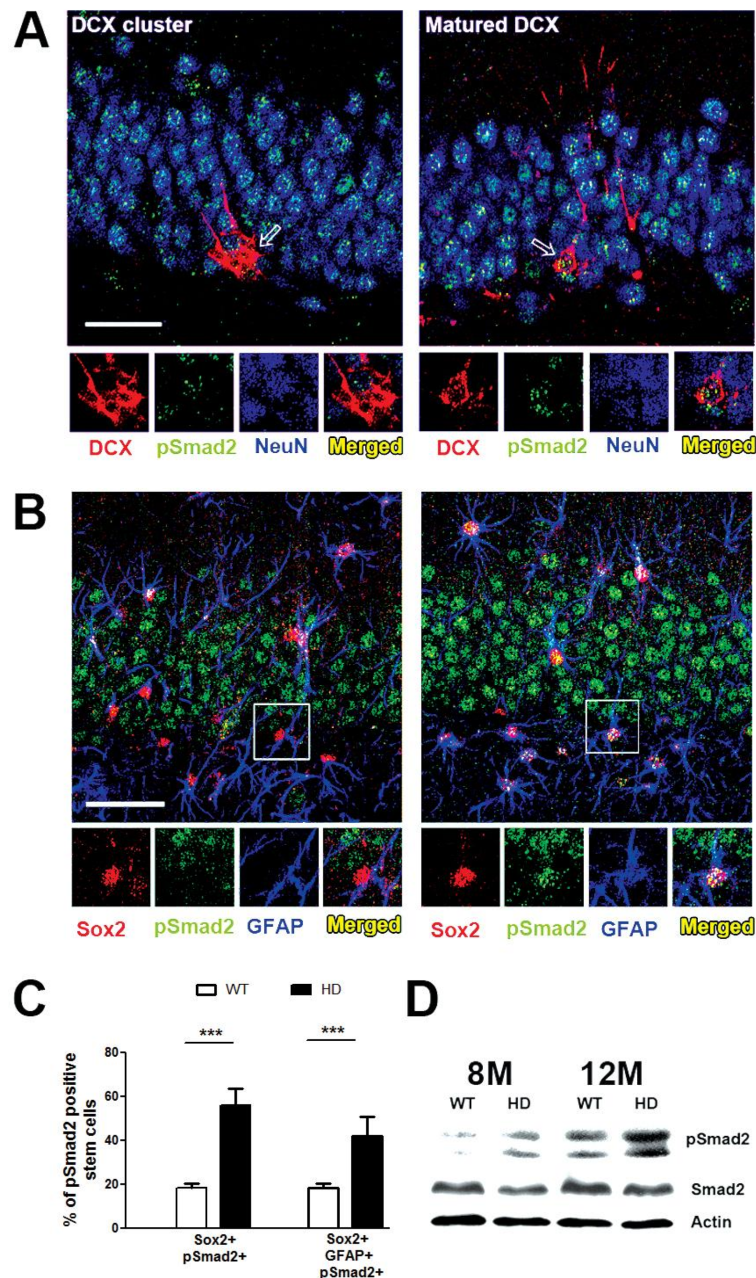

Fig .4.15. Enhanced TGF-beta1/Smad2 signaling in tgHD rats.
A) Presence of pSmad2 in 8 month-old WT DG granule cells. PSmad2 (green) is present in NeuN (blue) expressing cells and in mature DCX (red) positive cells (DCX cells in the granule cell layer presenting a dendritic tree perpendicular to the 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[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 [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).

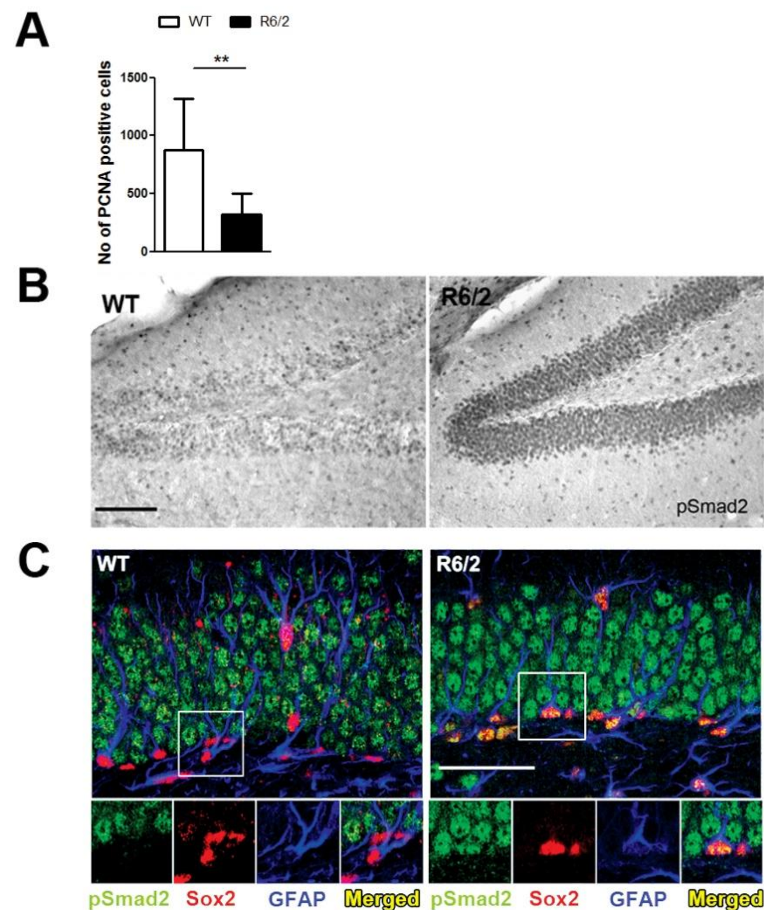

Fig.4.16. Reduced proliferation is associated with elevated TGF-beta1 signaling in R6/2 mice.
A) Quantitative analysis of the number of PCNA positive cells in the DG of 9 week-old WT and R6/2 mice. Data are expressed as means ± SD. For statistical analysis a Student t-test was performed. Note the reduction 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 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[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 [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[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.

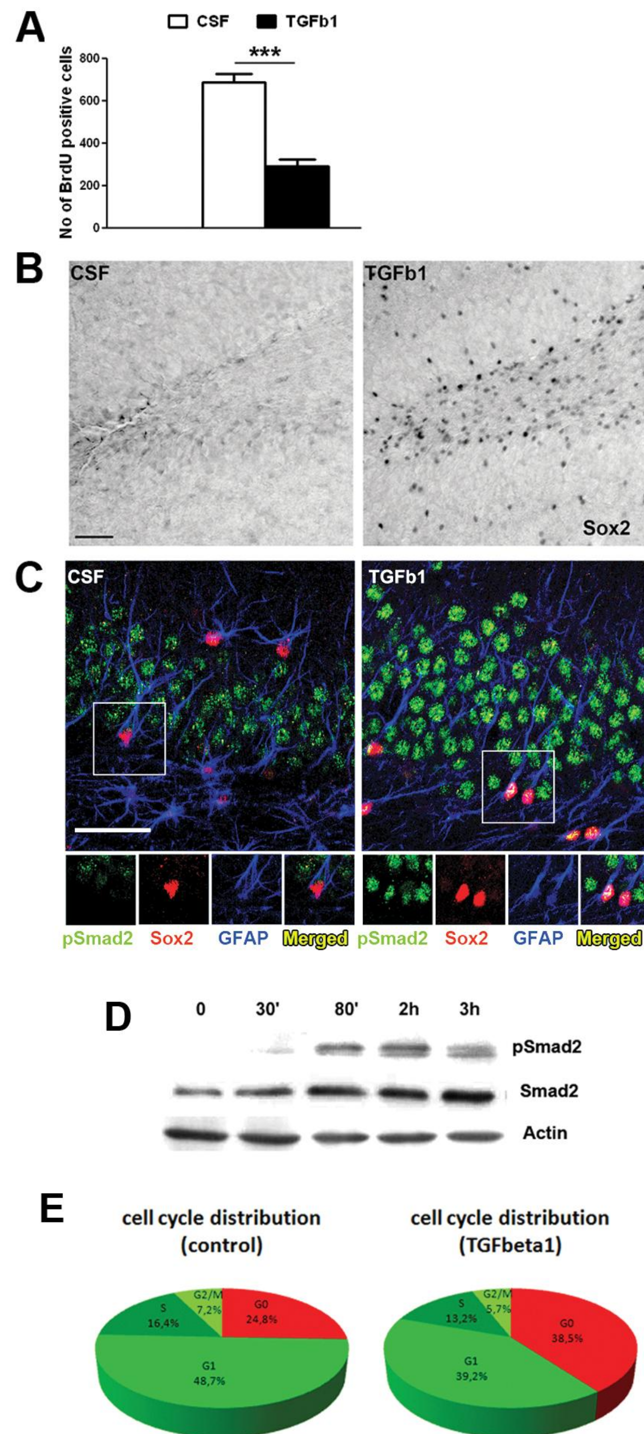

Fig.4.17. TGF-beta1 directed proliferation and cell cycle arrest is associated with enhanced SMAD2 signaling in stem cell niche.

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 G_0 (**$p < 0.01$). 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 immunohistrochemistry and Western bloting 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[85]. This suggest the presence of physiological TGF-beta1 levels in most brain areas[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 expresse 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.[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 [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 neurophere cultures derived from the adult rat hipocampus 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[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

increased resulting in a net gain of NeuN positive newly born neurons in the hippocampus of TGF-beta on mice. This is in agreement with data with previous reports showing increased neuronal differentiation and survival upon TGF-beta1 induction in the adult brain[93, 94]. Vice versa, increased neuronal cell death has been observed in TGF-beta1 knockout mice[167]. Importantly, TGF-beta1 has been involved in the generation of dopaminergic neurons in the developing brains[168].

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[98, 149, 169]. 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[71] .

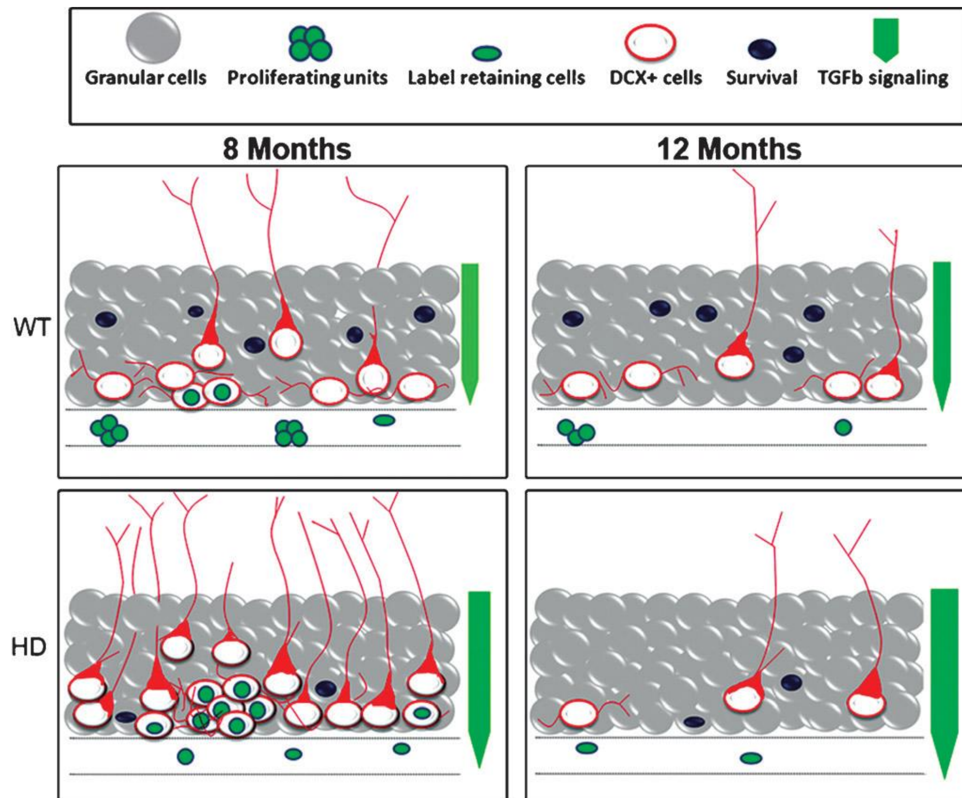

Fig.5.1. Schematic summary of modulation of neurogenesis at different cellular levels in the transgenic rat model of Huntington disease (tgHD).
In 8-month-old tgHD animals, the deficit in stem/progenitor proliferation was compensated by doublecortin (DCX)-positive neuroblasts (red cells) resulting in 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-proliverative 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 [98, 169, 170] in the A53T mutant alpha-synuclein mouse model of Parkinson's disease [171], in a mouse model of Alzheimer's disease, which expresses the human amyloid precursor protein (APP) with the V717F mutation (PDAPP mice)[172] and in the superoxide-dismutase 1 mouse model of Amyotrophic Lateral Sclerosis[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[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[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[94], and neural progenitors derived from HD mice demonstrate increased neuronal differentiation [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[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[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[58]. This context-dependency might explain also conflicting data reporting enhanced cell proliferation in the subventricular zone in HD patients[147] and diminished progenitor proliferation in the hippocampal neurogenic niche in HD animal models[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[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 [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 pre-requisite 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[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[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 trangenic 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.

7. Bibliography

1. Cajal: Degeneration and Regeneration of the Nervous System. Edited by J. D. Oxford, Oxford University Press, 1928, p

2. Caviness VS, Jr.: Time of neuron origin in the hippocampus and dentate gyrus of normal and reeler mutant mice: an autoradiographic analysis, J Comp Neurol 1973, 151:113-120

3. Caviness VS, Jr., Takahashi T, Nowakowski RS: Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model, Trends Neurosci 1995, 18:379-383

4. Altman J, Das GD: Autoradiographic and histologic evidence of postnatal neurogenesis in rats, The Journal of Comparative Neurology 1965, 124:319-335

5. Kaplan MS, Hinds JW: Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs, Science 1977, 197:1092-1094

6. Allen.E: The cessation of mitosis in the central nervous system of the albino rat, J Comp Neurol 1912, 22:547-568

7. Bryans WA: Mitotic activity in the brain of the adult rat, The Anatomical Record 1959, 133:65-73

8. Altman J, Das GD: Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats, J Comp Neurol 1965, 124:319-335

9. Nottebohm.F: Neuronal replacement in adulthood, Ann N Y Acad Sci 1985, 457:143-161

10. Cameron HA, Woolley CS, McEwen BS, Gould E: Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat, Neuroscience 1993, 56:337-344

11. Corotto FS, Henegar JA, Maruniak JA: Neurogenesis persists in the subependymal layer of the adult mouse brain, Neurosci Lett 1993, 149:111-114

12. Luskin MB: Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone, Neuron 1993, 11:173-189

13. Lois C, Alvarez-Buylla A: Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia, Proc Natl Acad Sci U S A 1993, 90:2074-2077

14. Lois C, Alvarez-Buylla A: Long-distance neuronal migration in the adult mammalian brain, Science 1994, 264:1145-1148

15. Kuhn HG, Fisher LJ, Taupin P, Gage FH: Factors influencing BrdU-labeling of cells in the adult brain, Soc Neurosci. Abstr. 1994, 20:671

16. van Praag H, Christie BR, Sejnowski TJ, Gage FH: Running enhances neurogenesis, learning, and long-term potentiation in mice, Proc Natl Acad Sci U S A 1999, 96:13427-13431.

17. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH: Effects of intracerebroventricular administration of EGF and FGF-2 on proliferation and differentiation of neural progenitors in adult rats: I. subventricular zone, striatum and olfactory bulb, Neurosci Abstr 1996, 22:980

18. Kaplan MS, Hinds JW: Gliogenesis of astrocytes and oligodendrocytes in the neocortical grey and white matter of the adult rat: electron microscopic analysis of light radioautographs, J Comp Neurol 1980, 193:711-727

19. Kuhn HG, Dickinson-Anson H, Gage FH: Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation, J Neurosci 1996, 16:2027-2033

20. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH: Neurogenesis in the adult human hippocampus, Nat Med 1998, 4:1313-1317

21. Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtas S, van Roon-Mom WM, Bjork-Eriksson T, Nordborg C, Frisen J, Dragunow M, Faull RL, Eriksson PS: Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension, Science 2007, 315:1243-1249

22. van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH: Functional neurogenesis in the adult hippocampus, Nature 2002, 415:1030-1034

23. Ming GL, Song H: Adult neurogenesis in the mammalian central nervous system, Annu Rev Neurosci 2005, 28:223-250

24. Morrison SJ, Shah NM, Anderson DJ: Regulatory mechanisms in stem cell biology, Cell 1997, 88:287-298

25. Gage FH: Mammalian neural stem cells, Science 2000, 287:1433-1438

26. Seki T, Arai Y: Temporal and spacial relationships between PSA-NCAM-expressing, newly generated granule cells, and radial glia-like cells in the adult dentate gyrus, J Comp Neurol 1999, 410:503-513

27. Carlen M, Cassidy RM, Brismar H, Smith GA, Enquist LW, Frisen J: Functional integration of adult-born neurons, Curr Biol 2002, 12:606-608

28. Stanfield BB, Trice JE: Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections, Exp Brain Res 1988, 72:399-406

29. Snyder EY, Yoon C, Flax JD, Macklis JD: Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex, Proc Natl Acad Sci U S A 1997, 94:11663-11668

30. Parent JM, Vexler ZS, Gong C, Derugin N, Ferriero DM: Rat forebrain neurogenesis and striatal neuron replacement after focal stroke, Ann Neurol 2002, 52:802-813

31. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH: Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain, J Neurosci 1997, 17:5820-5829

32. Song HJ, Stevens CF, Gage FH: Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons, Nat Neurosci 2002, 5:438-445

33. Toda H, Takahashi J, Mizoguchi A, Koyano K, Hashimoto N: Neurons generated from adult rat hippocampal stem cells form functional glutamatergic and GABAergic synapses in vitro, Exp Neurol 2000, 165:66-76.

34. Encinas JM, Vaahtokari A, Enikolopov G: Fluoxetine targets early progenitor cells in the adult brain, Proc Natl Acad Sci U S A 2006, 103:8233-8238

35. Petreanu L, Alvarez-Buylla A: Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction, J Neurosci 2002, 22:6106-6113

36. Betarbet R, Zigova T, Bakay RA, Luskin MB: Dopaminergic and GABAergic interneurons of the olfactory bulb are derived from the neonatal subventricular zone, Int J Dev Neurosci 1996, 14:921-930

37. McLean JH, Shipley MT: Postmitotic, postmigrational expression of tyrosine hydroxylase in olfactory bulb dopaminergic neurons, J Neurosci 1988, 8:3658-3669

38. Belluzzi O, Benedusi M, Ackman J, LoTurco JJ: Electrophysiological differentiation of new neurons in the olfactory bulb, J Neurosci 2003, 23:10411-10418

39. Song H, Kempermann G, Overstreet Wadiche L, Zhao C, Schinder AF, Bischofberger J: New neurons in the adult mammalian brain: synaptogenesis and functional integration, J Neurosci 2005, 25:10366-10368

40. Kempermann G, Kuhn HG, Gage FH: More hippocampal neurons in adult mice living in an enriched environment, Nature 1997, 386:493-495

41. Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS: Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory, J Neurobiol 1999, 39:569-578

42. Young D, Lawlor PA, Leone P, Dragunow M, During MJ: Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective, Nat Med 1999, 5:448-453

43. van Praag H, Kempermann G, Gage FH: Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus, Nature Neurosci 1999, 2:266-270

44. Darsalia V, Heldmann U, Lindvall O, Kokaia Z: Stroke-induced neurogenesis in aged brain, Stroke 2005, 36:1790-1795

45. Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH: Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus, J Neurosci 1997, 17:3727-3738

46. Couillard-Despres S, Wuertinger C, Kandasamy M, Caioni M, Stadler K, Aigner R, Bogdahn U, Aigner L: Ageing abolishes the effects of fluoxetine on neurogenesis, Mol Psychiatry 2009,

47. Gould E, McEwen BS, Tanapat P, Galea LAM: Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation, J Neurosci 1997, 17:2492-2498

48. Gould E, Tanapat P: Stress and hippocampal neurogenesis, Biol Psychiatry 1999, 46:1472-1479

49. Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E: Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress, Proc Natl Acad Sci U S A 1998, 95:3168-3171

50. Torner L, Karg S, Blume A, Kandasamy M, Kuhn HG, Winkler J, Aigner L, Neumann ID: Prolactin prevents chronic stress-induced decrease of adult hippocampal neurogenesis and promotes neuronal fate, J Neurosci 2009, 29:1826-1833

51. Govindaraj A JCP: Transforming Growth Factor-ß Signaling

in Bladder Fibrosis, ARBS Annual Review of Biomedical Sciences 2008, 10:1-5

52. Trejo JL, Carro E, Torres-Aleman I: Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus, J Neurosci 2001, 21:1628-1634

53. Aberg ND, Johansson I, Aberg MA, Lind J, Johansson UE, Cooper-Kuhn CM, Kuhn HG, Isgaard J: Peripheral administration of GH induces cell proliferation in the brain of adult hypophysectomized rats, J Endocrinol 2009, 201:141-150

54. Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA: Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo, Proc Natl Acad Sci U S A 2002, 99:11946-11950

55. Jung KH, Chu K, Lee ST, Kim SJ, Sinn DI, Kim SU, Kim M, Roh JK: Granulocyte colony-stimulating factor stimulates neurogenesis via vascular endothelial growth factor with STAT activation, Brain Res 2006, 1073-1074:190-201

56. Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A: Noggin antagonizes BMP signaling to create a niche for adult neurogenesis, Neuron 2000, 28:713-726

57. Massague J: TGF-beta signal transduction, Annu Rev Biochem 1998, 67:753-791

58. Aigner L, Bogdahn U: TGF-beta in neural stem cells and in tumors of the central nervous system, Cell Tissue Res 2008, 331:225-241

59. Moses HL, Branum EL, Proper JA, Robinson RA: Transforming growth factor production by chemically transformed cells, Cancer Res 1981, 41:2842-2848

60. Roberts AB: Molecular and cell biology of TGF-beta, Miner Electrolyte Metab 1998, 24:111-119

61. Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB: New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues, Proc Natl Acad Sci U S A 1981, 78:5339-5343

62. Annes JP, Munger JS, Rifkin DB: Making sense of latent TGFbeta activation, J Cell Sci 2003, 116:217-224

63. Bottner M, Krieglstein K, Unsicker K: The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions, J Neurochem 2000, 75:2227-2240

64. Dennler S, Goumans MJ, ten Dijke P: Transforming growth factor beta signal transduction, J Leukoc Biol 2002, 71:731-740

65. Massague J: How cells read TGF-beta signals, Nat Rev Mol Cell Biol 2000, 1:169-178

66. Massague J, Gomis RR: The logic of TGFbeta signaling, FEBS Lett 2006, 580:2811-2820

67. Massague J, Wotton D: Transcriptional control by the TGF-beta/Smad signaling system, Embo J 2000, 19:1745-1754

68. Massague J, Chen YG: Controlling TGF-beta signaling, Genes Dev 2000, 14:627-644

69. Bottner M, Unsicker K, Suter-Crazzolara C: Expression of TGF-beta type II receptor mRNA in the CNS, Neuroreport 1996, 7:2903-2907

70. Unsicker K, Flanders KC, Cissel DS, Lafyatis R, Sporn MB: Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system, Neuroscience 1991, 44:613-625

71. Bode FJ, Stephan M, Suhling H, Pabst R, Straub RH, Raber KA, Bonin M, Nguyen HP, Riess O, Bauer A, Sjoberg C, Petersen A, von Horsten S: Sex differences in a transgenic rat model of Huntington's disease: decreased 17beta-estradiol levels correlate with reduced numbers of DARPP32+ neurons in males, Hum Mol Genet 2008, 17:2595-2609

72. Wyss-Coray T: Tgf-Beta pathway as a potential target in neurodegeneration and Alzheimer's, Curr Alzheimer Res 2006, 3:191-195

73. Ilzecka J, Stelmasiak Z, Dobosz B: Transforming growth factor-Beta 1 (tgf-Beta 1) in patients with amyotrophic lateral sclerosis, Cytokine 2002, 20:239-243

74. Jason GW, Suchowersky O, Pajurkova EM, Graham L, Klimek ML, Garber AT, Poirier-Heine D: Cognitive manifestations of Huntington disease in relation to genetic structure and clinical onset, Arch Neurol 1997, 54:1081-1088

75. Flanders KC, Ren RF, Lippa CF: Transforming growth factor-betas in neurodegenerative disease, Prog Neurobiol 1998, 54:71-85

76. Ueberham U, Ueberham E, Gruschka H, Arendt T: Connective tissue growth factor in Alzheimer's disease, Neuroscience 2003, 116:1-6

77. Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS, Berger WH: Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors, Cancer Res 1987, 47:707-712

78. Jachimczak P, Bogdahn U, Schneider J, Behl C, Meixensberger J, Apfel R, Dorries R, Schlingensiepen KH, Brysch W: The effect of transforming growth factor-beta 2-specific phosphorothioate-anti-sense oligodeoxynucleotides in reversing cellular immunosuppression in malignant glioma, J Neurosurg 1993, 78:944-951

79. Kjellman C, Olofsson SP, Hansson O, Von Schantz T, Lindvall M, Nilsson I, Salford LG, Sjogren HO, Widegren B: Expression of TGF-beta isoforms, TGF-beta

receptors, and SMAD molecules at different stages of human glioma, Int J Cancer 2000, 89:251-258

80. Wick W, Platten M, Weller M: Glioma cell invasion: regulation of metalloproteinase activity by TGF-beta, J Neurooncol 2001, 53:177-185

81. Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, Hau P, Bogdahn U, Fischer-Blass B, Jachimczak P: Targeted tumor therapy with the TGF-beta2 antisense compound AP 12009, Cytokine Growth Factor Rev 2006, 17:129-139

82. Wachs FP, Winner B, Couillard-Despres S, Schiller T, Aigner R, Winkler J, Bogdahn U, Aigner L: Transforming growth factor-beta1 is a negative modulator of adult neurogenesis, J Neuropathol Exp Neurol 2006, 65:358-370

83. Wyss-Coray T, Mucke L: Inflammation in neurodegenerative disease--a double-edged sword, Neuron 2002, 35:419-432

84. Wyss-Coray T, Feng L, Masliah E, Ruppe MD, Lee HS, Toggas SM, Rockenstein EM, Mucke L: Increased central nervous system production of extracellular matrix components and development of hydrocephalus in transgenic mice overexpressing transforming growth factor-beta 1, Am J Pathol 1995, 147:53-67

85. Lin AH, Luo J, Mondshein LH, ten Dijke P, Vivien D, Contag CH, Wyss-Coray T: Global analysis of Smad2/3-dependent TGF-beta signaling in living mice reveals prominent tissue-specific responses to injury, J Immunol 2005, 175:547-554

86. Unsicker K, Krieglstein K: TGF-betas and their roles in the regulation of neuron survival, Adv Exp Med Biol 2002, 513:353-374

87. Krieglstein K, Strelau J, Schober A, Sullivan A, Unsicker K: TGF-beta and the regulation of neuron survival and death, J Physiol Paris 2002, 96:25-30

88. Konig HG, Kogel D, Rami A, Prehn JH: TGF-{beta}1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa}B signaling, J Cell Biol 2005, 168:1077-1086

89. Vergeli M, mazzanti B, Ballerini C, Gran B, Amaducci L, Massacesi L: Transforming growth factor-beta 1 inhibits the proliferation of rat astrocytes induced by serum and growth factors, J Neurosci Res 1995, 40:127-133

90. Suzumura A, Sawada M, Yamamoto H, Marunouchi T: Transforming growth factor-beta suppresses activation and proliferation of microglia in vitro, J Immunol 1993, 151:2150-2158

91. Buckwalter MS, Yamane M, Coleman BS, Ormerod BK, Chin JT, Palmer T, Wyss-Coray T: Chronically increased transforming growth factor-beta1 strongly inhibits hippocampal neurogenesis in aged mice, Am J Pathol 2006, 169:154-164

92. Mathieu P, Piantanida AP, Pitossi F: Chronic expression of transforming growth factor-beta enhances adult neurogenesis, Neuroimmunomodulation 2010, 17:200-201

93. Ma M, Ma Y, Yi X, Guo R, Zhu W, Fan X, Xu G, Frey WH, 2nd, Liu X: Intranasal delivery of transforming growth factor-beta1 in mice after stroke reduces infarct volume and increases neurogenesis in the subventricular zone, BMC Neurosci 2008, 9:117

94. Battista D, Ferrari CC, Gage FH, Pitossi FJ: Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus, Eur J Neurosci 2006, 23:83-93

95. Dhandapani KM, Brann DW: Transforming growth factor-beta: a neuroprotective factor in cerebral ischemia, Cell Biochem Biophys 2003, 39:13-22

96. Ueberham U, Ueberham E, Gruschka H, Arendt T: Altered subcellular location of phosphorylated Smads in Alzheimer's disease, Eur J Neurosci 2006, 24:2327-2334

97. Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, Hirsch EC: Dopamine depletion impairs precursor cell proliferation in Parkinson disease, Nat Neurosci 2004, 7:726-735

98. Gil JM, Mohapel P, Araujo IM, Popovic N, Li JY, Brundin P, Petersen A: Reduced hippocampal neurogenesis in R6/2 transgenic Huntington's disease mice, Neurobiol Dis 2005, 20:744-751

99. Demars M, Hu YS, Gadadhar A, Lazarov O: Impaired neurogenesis is an early event in the etiology of familial Alzheimer's disease in transgenic mice, J Neurosci Res 2010, 88:2103-2117

100. The Huntington's Disease Collaborative Research Group: The Huntington's Disease Collaborative Research Group, A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. , Cell 1993, 72:971-983

101. Walker FO: Huntington's disease, Lancet 2007, 369:218-228

102. Wells RD, Dere R, Hebert ML, Napierala M, Son LS: Advances in mechanisms of genetic instability related to hereditary neurological diseases, Nucleic Acids Res 2005, 33:3785-3798

103. Myers RH MK, MacDonald ME.: Genetic instabilities and hereditary neurological diseases (Warren ST, Wells RT, eds), New York: Academic Press 1998, 301–323

104. Rosenberg NK, Sorensen SA, Christensen AL: Neuropsychological characteristics of Huntington's disease carriers: a double blind study, J Med Genet 1995, 32:600-604

105. Berardelli A, Noth J, Thompson PD, Bollen EL, Curra A, Deuschl G, van Dijk JG, Topper R, Schwarz M, Roos RA: Pathophysiology of chorea and bradykinesia in Huntington's disease, Mov Disord 1999, 14:398-403

106. Hayden MR, Berkowicz AL, Beighton PH, Yiptong C: Huntington's chorea on the island of Mauritius, S Afr Med J 1981, 60:1001-1002

107. Hunt VP, Walker FO: Dysphagia in Huntington's disease, J Neurosci Nurs 1989, 21:92-95

108. Lavers A: Feeding difficulties in patients with Huntington's chorea, Nurs Times 1982, 78:920-921

109. Quinn N, Schrag A: Huntington's disease and other choreas, J Neurol 1998, 245:709-716

110. R A Roos JH, M Vegter-van der Vlis, G J van Ommen, G W Bruyn: Duration of illness in Huntington's disease is not related to age at onset, J Neurol Neurosurg Psychiatry 1993, 98-100

111. Stephan Claes MKVZ, MS; Erik Legius, MD, PhD; René Dom, MD, PhD; Michel Malfroid, MD; Franz Baro, MD, PhD; Jan Godderis, MD, PhD; Jean-Jacques Cassiman, MD, PhD: Correlations Between Triplet Repeat Expansion and Clinical Features in Huntington's Disease, Arch Neurol 1995, 749-753

112. Harper PS: The epidemiology of Huntington's disease, Human Genetics 1964, 365-376

113. Squitieri F, Andrew SE, Goldberg YP, Kremer B, Spence N, Zeisler J, Nichol K, Theilmann J, Greenberg J, Goto J, et al.: DNA haplotype analysis of Huntington

disease reveals clues to the origins and mechanisms of CAG expansion and reasons for geographic variations of prevalence, Hum Mol Genet 1994, 3:2103-2114

114. Okun MS, Thommi N: Americo Negrette (1924 to 2003): diagnosing Huntington disease in Venezuela, Neurology 2004, 63:340-343

115. Gusella JF, Wexler NS, Conneally PM, Naylor SL, Anderson MA, Tanzi RE, Watkins PC, Ottina K, Wallace MR, Sakaguchi AY, et al.: A polymorphic DNA marker genetically linked to Huntington's disease, Nature 1983, 306:234-238

116. Bonilla E: [Huntington disease. A review], Invest Clin 2000, 41:117-141

117. Schmitt I, Bachner D, Megow D, Henklein P, Hameister H, Epplen JT, Riess O: Expression of the Huntington disease gene in rodents: cloning the rat homologue and evidence for downregulation in non-neuronal tissues during development, Hum Mol Genet 1995, 4:1173-1182

118. Landwehrmeyer GB, McNeil SM, Dure LSt, Ge P, Aizawa H, Huang Q, Ambrose CM, Duyao MP, Bird ED, Bonilla E, et al.: Huntington's disease gene: regional and cellular expression in brain of normal and affected individuals, Ann Neurol 1995, 37:218-230

119. Nasir J, Floresco SB, O'Kusky JR, Diewert VM, Richman JM, Zeisler J, Borowski A, Marth JD, Phillips AG, Hayden MR: Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes, Cell 1995, 81:811-823

120. Dragatsis I, Levine MS, Zeitlin S: Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice, Nat Genet 2000, 26:300-306

121. Hansson O, Petersen A, Leist M, Nicotera P, Castilho RF, Brundin P: Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity, Proc Natl Acad Sci U S A 1999, 96:8727-8732

122. Reiner A, Dragatsis I, Zeitlin S, Goldowitz D: Wild-type huntingtin plays a role in brain development and neuronal survival, Mol Neurobiol 2003, 28:259-276

123. Himmelbauer H, Wedemeyer N, Haaf T, Wanker EE, Schalkwyk LC, Lehrach H: IRS-PCR-based genetic mapping of the huntingtin interacting protein gene (HIP1) on mouse chromosome 5, Mamm Genome 1998, 9:26-31

124. Karpuj MV, Garren H, Slunt H, Price DL, Gusella J, Becher MW, Steinman L: Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei, Proc Natl Acad Sci U S A 1999, 96:7388-7393

125. Giampa C, DeMarch Z, D'Angelo V, Morello M, Martorana A, Sancesario G, Bernardi G, Fusco FR: Striatal modulation of cAMP-response-element-binding protein (CREB) after excitotoxic lesions: implications with neuronal vulnerability in Huntington's disease, Eur J Neurosci 2006, 23:11-20

126. Giuliano P, De Cristofaro T, Affaitati A, Pizzulo GM, Feliciello A, Criscuolo C, De Michele G, Filla A, Avvedimento EV, Varrone S: DNA damage induced by polyglutamine-expanded proteins, Hum Mol Genet 2003, 12:2301-2309

127. Hermel E, Gafni J, Propp SS, Leavitt BR, Wellington CL, Young JE, Hackam AS, Logvinova AV, Peel AL, Chen SF, Hook V, Singaraja R, Krajewski S, Goldsmith PC, Ellerby HM, Hayden MR, Bredesen DE, Ellerby LM: Specific caspase interactions and amplification are involved in selective neuronal vulnerability in Huntington's disease, Cell Death Differ 2004, 11:424-438

128. Lee WC, Yoshihara M, Littleton JT: Cytoplasmic aggregates trap polyglutamine-containing proteins and block axonal transport in a Drosophila model of Huntington's disease, Proc Natl Acad Sci U S A 2004, 101:3224-3229

129. Steffan JS, Kazantsev A, Spasic-Boskovic O, Greenwald M, Zhu YZ, Gohler H, Wanker EE, Bates GP, Housman DE, Thompson LM: The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription, Proc Natl Acad Sci U S A 2000, 97:6763-6768

130. Chesselet MF, Delfs JM, Mackenzie L: Dopamine control of gene expression in basal ganglia nuclei: striatal and nonstriatal mechanisms, Adv Pharmacol 1998, 42:674-677

131. Robitaille Y, Lopes-Cendes I, Becher M, Rouleau G, Clark AW: The neuropathology of CAG repeat diseases: review and update of genetic and molecular features, Brain Pathol 1997, 7:901-926

132. Nguyen HP, Kobbe P, Rahne H, Worpel T, Jager B, Stephan M, Pabst R, Holzmann C, Riess O, Korr H, Kantor O, Petrasch-Parwez E, Wetzel R, Osmand A, von Horsten S: Behavioral abnormalities precede neuropathological markers in rats transgenic for Huntington's disease, Hum Mol Genet 2006, 15:3177-3194

133. Osmand AP, Berthelier V, Wetzel R: Imaging polyglutamine deposits in brain tissue, Methods Enzymol 2006, 412:106-122

134. Bates G HP, Jones L: Huntington's Disease, Oxford University Press, New York 2002,

135. von Horsten S, Schmitt I, Nguyen HP, Holzmann C, Schmidt T, Walther T, Bader M, Pabst R, Kobbe P, Krotova J, Stiller D, Kask A, Vaarmann A, Rathke-Hartlieb S, Schulz JB, Grasshoff U, Bauer I, Vieira-Saecker AM, Paul M, Jones L, Lindenberg KS, Landwehrmeyer B, Bauer A, Li XJ, Riess O: Transgenic rat model of Huntington's disease, Hum Mol Genet 2003, 12:617-624

136. Rosas HD, Koroshetz WJ, Chen YI, Skeuse C, Vangel M, Cudkowicz ME, Caplan K, Marek K, Seidman LJ, Makris N, Jenkins BG, Goldstein JM: Evidence for more widespread cerebral pathology in early HD: an MRI-based morphometric analysis, Neurology 2003, 60:1615-1620

137. Kassubek J, Gaus W, Landwehrmeyer GB: Evidence for more widespread cerebral pathology in early HD: an MRI-based morphometric analysis, Neurology 2004, 62:523-524; author reply 524

138. Beal MF, Ferrante RJ, Swartz KJ, Kowall NW: Chronic quinolinic acid lesions in rats closely resemble Huntington's disease, J Neurosci 1991, 11:1649-1659

139. Miller PJ, Zaborszky L: 3-Nitropropionic acid neurotoxicity: visualization by silver staining and implications for use as an animal model of Huntington's disease, Exp Neurol 1997, 146:212-229

140. Zeron MM, Hansson O, Chen N, Wellington CL, Leavitt BR, Brundin P, Hayden MR, Raymond LA: Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease, Neuron 2002, 33:849-860

141. Faber PW, Alter JR, MacDonald ME, Hart AC: Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron, Proc Natl Acad Sci U S A 1999, 96:179-184

142. van Ham TJ, Breitling R, Swertz MA, Nollen EA: Neurodegenerative diseases: Lessons from genome-wide screens in small model organisms, EMBO Mol Med 2009, 1:360-370

143. Shulman JM, Shulman LM, Weiner WJ, Feany MB: From fruit fly to bedside: translating lessons from Drosophila models of neurodegenerative disease, Curr Opin Neurol 2003, 16:443-449

144. Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trottier Y, Lehrach H, Davies SW, Bates GP: Exon 1 of the HD gene with

an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice, Cell 1996, 87:493-506

145. Petrasch-Parwez E, Nguyen HP, Lobbecke-Schumacher M, Habbes HW, Wieczorek S, Riess O, Andres KH, Dermietzel R, Von Horsten S: Cellular and subcellular localization of Huntingtin [corrected] aggregates in the brain of a rat transgenic for Huntington disease, J Comp Neurol 2007, 501:716-730

146. Tattersfield AS, Croon RJ, Liu YW, Kells AP, Faull RL, Connor B: Neurogenesis in the striatum of the quinolinic acid lesion model of Huntington's disease, Neuroscience 2004, 127:319-332

147. Curtis MA, Penney EB, Pearson AG, van Roon-Mom WM, Butterworth NJ, Dragunow M, Connor B, Faull RL: Increased cell proliferation and neurogenesis in the adult human Huntington's disease brain, Proc Natl Acad Sci U S A 2003, 100:9023-9027

148. Ueberham U, Ueberham E, Bruckner MK, Seeger G, Gartner U, Gruschka H, Gebhardt R, Arendt T: Inducible neuronal expression of transgenic TGF-beta1 in vivo: dissection of short-term and long-term effects, Eur J Neurosci 2005, 22:50-64

149. Kohl Z, Kandasamy M, Winner B, Aigner R, Gross C, Couillard-Despres S, Bogdahn U, Aigner L, Winkler J: Physical activity fails to rescue hippocampal neurogenesis deficits in the R6/2 mouse model of Huntington's disease, Brain Res 2007, 1155:24-33

150. Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, Aigner L: Doublecortin expression levels in adult brain reflect neurogenesis, European Journal of Neuroscience 2005, 21:1-14

151. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Moller A, Nielsen K, Nyengaard JR, Pakkenberg B, et al.: The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis, Apmis 1988, 96:857-881

152. Williams RW, Rakic P: Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material, J Comp Neurol 1988, 278:344-352

153. Seki T, Namba T, Mochizuki H, Onodera M: Clustering, migration, and neurite formation of neural precursor cells in the adult rat hippocampus, J Comp Neurol 2007, 502:275-290

154. Wachs FP, Couillard-Despres S, Engelhardt M, Wilhelm D, Ploetz S, Vroemen M, Kaesbauer J, Uyanik G, Klucken J, Karl C, Tebbing J, Svendsen C, Weidner N, Kuhn HG, Winkler J, Aigner L: High Efficacy of Clonal Growth and Expansion of Adult Neural Stem Cells, Lab Invest 2003, 83:949-962

155. Endl E, Steinbach P, Knuchel R, Hofstadter F: Analysis of cell cycle-related Ki-67 and p120 expression by flow cytometric BrdUrd-Hoechst/7AAD and immunolabeling technique, Cytometry 1997, 29:233-241

156. Van Raamsdonk JM, Pearson J, Rogers DA, Bissada N, Vogl AW, Hayden MR, Leavitt BR: Loss of wild-type huntingtin influences motor dysfunction and survival in the YAC128 mouse model of Huntington disease, Hum Mol Genet 2005, 14:1379-1392

157. Ciamei A, Morton AJ: Rigidity in social and emotional memory in the R6/2 mouse model of Huntington's disease, Neurobiol Learn Mem 2008, 89:533-544

158. Bizon JL, Lee HJ, Gallagher M: Neurogenesis in a rat model of age-related cognitive decline, Aging Cell 2004, 3:227-234

159. Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ: A functional role for adult hippocampal neurogenesis in spatial pattern separation, Science 2009, 325:210-213

160. Jessberger S, Clark RE, Broadbent NJ, Clemenson GD, Jr., Consiglio A, Lie DC, Squire LR, Gage FH: Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats, Learn Mem 2009, 16:147-154

161. Hernandez-Rabaza V, Llorens-Martin M, Velazquez-Sanchez C, Ferragud A, Arcusa A, Gumus HG, Gomez-Pinedo U, Perez-Villalba A, Rosello J, Trejo JL, Barcia JA, Canales JJ: Inhibition of adult hippocampal neurogenesis disrupts contextual learning but spares spatial working memory, long-term conditional rule retention and spatial reversal, Neuroscience 2009, 159:59-68

162. Walton M, Woodgate AM, Muravlev A, Xu R, During MJ, Dragunow M: CREB phosphorylation promotes nerve cell survival, J Neurochem 1999, 73:1836-1842

163. Jagasia R, Steib K, Englberger E, Herold S, Faus-Kessler T, Saxe M, Gage FH, Song H, Lie DC: GABA-cAMP response element-binding protein signaling regulates maturation and survival of newly generated neurons in the adult hippocampus, J Neurosci 2009, 29:7966-7977

164. Miller MW: Expression of transforming growth factor-beta in developing rat cerebral cortex: effects of prenatal exposure to ethanol, J Comp Neurol 2003, 460:410-424

165. Vincze C, Pal G, Wappler EA, Szabo ER, Nagy ZG, Lovas G, Dobolyi A: Distribution of mRNAs encoding transforming growth factors-beta1, -2, and -3 in the intact rat brain and after experimentally induced focal ischemia, J Comp Neurol 518:3752-3770

166. Siegenthaler JA, Miller MW: Transforming growth factor beta 1 promotes cell cycle exit through the cyclin-dependent kinase inhibitor p21 in the developing cerebral cortex, J Neurosci 2005, 25:8627-8636

167. Brionne TC, Tesseur I, Masliah E, Wyss-Coray T: Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain, Neuron 2003, 40:1133-1145

168. Roussa E, Farkas LM, Krieglstein K: TGF-beta promotes survival on mesencephalic dopaminergic neurons in cooperation with Shh and FGF-8, Neurobiol Dis 2004, 16:300-310

169. Lazic SE, Grote H, Armstrong RJ, Blakemore C, Hannan AJ, van Dellen A, Barker RA: Decreased hippocampal cell proliferation in R6/1 Huntington's mice, Neuroreport 2004, 15:811-813

170. Phillips W, Morton AJ, Barker RA: Abnormalities of neurogenesis in the R6/2 mouse model of Huntington's disease are attributable to the in vivo microenvironment, J Neurosci 2005, 25:11564-11576

171. Winner B, Couillard-Despres S, Geyer M, Aigner R, Bogdahn U, Aigner L, Kuhn HG, Winkler J: Dopaminergic lesion enhances growth factor-induced striatal neuroblast migration, J Neuropathol Exp Neurol 2008, 67:105-116

172. Donovan MH, Yazdani U, Norris RD, Games D, German DC, Eisch AJ: Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease, J Comp Neurol 2006, 495:70-83

173. Liu Z, Martin LJ: The adult neural stem and progenitor cell niche is altered in amyotrophic lateral sclerosis mouse brain, J Comp Neurol 2006, 497:468-488

174. Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA: Evidence for stroke-induced neurogenesis in the human brain, Proc Natl Acad Sci U S A 2006, 103:13198-13202

175. Kokaia Z, Thored P, Arvidsson A, Lindvall O: Regulation of stroke-induced neurogenesis in adult brain--recent scientific progress, Cereb Cortex 2006, 16 Suppl 1:i162-167

176. Lorincz MT, Zawistowski VA: Expanded CAG repeats in the murine Huntington's disease gene increases neuronal differentiation of embryonic and neural stem cells, Mol Cell Neurosci 2009, 40:1-13

177. Das S, Basu A: Inflammation: a new candidate in modulating adult neurogenesis, J Neurosci Res 2008, 86:1199-1208

178. Whitney NP, Eidem TM, Peng H, Huang Y, Zheng JC: Inflammation mediates varying effects in neurogenesis: relevance to the pathogenesis of brain injury and neurodegenerative disorders, J Neurochem 2009, 108:1343-1359

179. Chabanon A, Desterke C, Rodenburger E, Clay D, Guerton B, Boutin L, Bennaceur-Griscelli A, Pierre-Louis O, Uzan G, Abecassis L, Bourgeade MF, Lataillade JJ, Le Bousse-Kerdiles MC: A cross-talk between stromal cell-derived factor-1 and transforming growth factor-beta controls the quiescence/cycling switch of CD34(+) progenitors through FoxO3 and mammalian target of rapamycin, Stem Cells 2008, 26:3150-3161

180. Ficara F, Murphy MJ, Lin M, Cleary ML: Pbx1 regulates self-renewal of long-term hematopoietic stem cells by maintaining their quiescence, Cell Stem Cell 2008, 2:484-496

181. Shen Z, Chen L, Hao F, Wu J: Transcriptional regulation of Foxp3 gene: Multiple signal pathways on the road, Med Res Rev 2009

Publications

1) ***Brain Research 2007***
   Physical activity fails to rescue hippocampal neurogenesis deficits in the R6/2 mouse model of Huntington's disease
   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***
   Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors.
   Francisco J. Rivera, **Mahesh Kandasamy**, Sebastien Couillard-Despres, Massimiliano Caioni, Rosario Sanchez, Christophe Huber, Norbert Weidner, Ulrich Bogdahn, Ludwig Aigner

3) ***European Journal of Neuroscience 2009***
   Changes in adult olfactory bulb neurogenesis in mice expressing the A30P mutant form of alpha synuclein
   Franz Marxreiter, Silke Nuber, **Mahesh Kandasamy**, Jochen Klucken, Robert Aigner, Ralf Burgmayer, Sebastien Couillard-Despres, Olaf Riess, Jürgen Winkler and Beate Winner

4) ***Molecular Psychiatry 2009***
   Ageing Abolishes the Effects of Fluoxetine on Neurogenesis
   Sebastien Couillard-Despres, Christoph Wuertinger, **Mahesh Kandasamy**, Massimiliano Caioni, Katrin Altendorfer, Robert Aigner, Ulrich Bogdahn, Ludwig Aigner

5) ***Journal of Neuroscience 2009***
   Prolactin prevents chronic stress induced decrease of adult hippocampal neurogenesis and promotes neuronal fate.
   Luz Torner, Sandra Karg, Annegret Blume, **Mahesh Kandasamy**, Hans-Georg Kuhn, Juergen Winkler, Ludwig Aigner, Inga D. Neumann

6) ***Cell Physiol Biochem 2009***
   Mesenchymal stem cells promote oligodendroglial differentiation in hippocampal slice cultures.
   Rivera FJ*, Siebzehnrubl FA*, **Kandasamy M***, Couillard-Despres S, Caioni M, Poehler AM, Berninger B, Sandner B, Bogdahn U, Goetz M, Bluemcke I, Weidner N, Aigner L.
   (*equal contribution)

7) ***Stem Cells Dev 2010 (Review article)***
   Deciphering The Oligodendrogenic Program Of Neural Progenitors: Cell Intrinsic And Extrinsic Regulators.

Rivera FJ, Steffenhagen C, Kremer D, **Kandasamy M,** Sandner B, Couillard-Despres S, Weidner N, Kuery P, Aigner L.
(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
Mahesh Kandasamy, Sebastien Couillard-Despres, Kerstin A. Raber, Michael Stephan, Bernadette Lehner, Beate Winner, MD, Zacharias Kohl, Francisco J. Rivera, Huu Phuc Nguyen, Olaf Riess, Ulrich Bogdahn, Jürgen Winkler, Stephan von Hörsten, and Ludwig Aigner.

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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