Intracellular signaling pathways in the dopaminergic specification of fetal mesencephalic neural stem cells

by

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“All science is philosophy, whether it knows and wills it or not.”

Martin Heidegger

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Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Meine Person betreffend erkläre ich hiermit, daß keine früheren erfolglosen Promotionsverfahren stattgefunden haben.

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List of Abbreviations

ATP	Adenosine triphosphate
bp	Base pairs
cAMP	Cycloadenosine monophosphate
CNS	Central nervous system
DAPI	4’, 6’-Diamidino-2-Phenylindol (nuclear stain)
DNA	Desoxyribonucleic acid
dNTPs	Desoxynucleotide triphosphates
E	Embryonic day (e.g. E14)
Egf	Epidermal growth factor
Egf-R	Epidermal growth factor receptor
ES cells	Embryonic stem cells
FACS	Flourescence Activated Cell Sorting
Fgf	Fibroblast growth factor
GFP	Green Fluorescent Protein
ICM	Inner cell mass
IL-1	Interleukin-1
IL-1RI	Interleukin-1 receptor type I
MAPK	Mitogen-activated protein kinase
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PCR	Polymerase chain reaction
PD	Parkinson’s disease
qRT-PCR	Quantitative reverse transcription (real-time) PCR
RNA	Ribonucleic acid
Rcf	Relative centrifugal force (=1.12$\times$ radius of rotor (rpm/1000)^2)
Rpm	Rounds per minute
RT	Reverse transcriptase
Shh	Sonic hedgehog
SVZ	Subventricular zone
Th	Tyrosine hydroxylase
VZ	Ventricular zone

Summary

Neural stem (progenitor) cells (NPCs) from fetal tissue are an ideal transplantable cell source. They divide rapidly, are able to generate cells of all three neural lineages and do not divide uncontrolled once transplanted into a host organism. To obtain large quantities of cells for transplantation strategies and to eliminate primary cell contaminations, long periods of *in vitro* cultivation are necessary.

Mouse NPCs are a crucial tool for further investigations of neural stem cells because they make the employment of transgenic animals *in vivo* and cells *in vitro* possible. So far only short-term expanded fetal mouse NPCs have been shown to generate dopaminergic neurons and it is not clear whether this was due to differentiation or a result of increased survival of primary dopaminergic neurons.

The aims of the thesis were to characterize mouse fetal NPCs, to establish the long-term expansion of fetal mouse NPCs and the generation of dopaminergic neurons in long-term expanded fetal mouse NPCs, to investigate the signaling mechanisms involved in the differentiation of mouse fetal NPCs towards the dopaminergic phenotype and to compare short and long-term expanded NPCs.

Long-term expanded fetal mesencephalic NPCs could be grown under suspension and adherent culture conditions and showed self-renewing capacity as well as markers typical for NPCs. They could be differentiated into the three major cell types of the nervous system, but suspension NPCs had a larger potential to generate neurons than adherently grown NPCs. Signaling cascades involved in this process were p38 and Erk1/2 mediated. Long-term expanded NPCs did not have the potential to generate neuronal sub-types. Importantly, they did not generate dopaminergic neurons. Mouse fetal NPCs from three different developmental stages (E10, E12, and E14) were employed but were not able to differentiate into dopaminergic neurons using factors known to stimulate *in vitro* dopaminergic specification.

When cultivated *in vitro* for short periods, fetal mesencephalic NPCs were able to generate dopaminergic neurons. By eliminating all primary Th-positive neurons, FACS-sorting of NPCs proved a *de novo* generation of dopaminergic neurons, because after cultivation and differentiation of Th-depleted cell solutions dopaminergic neurons were present in the culture. However, these newly generated neurons failed to incorporate BrdU, making a generation without cell division from precursors probable. The precursor population of short cultures differed from long-term expanded cultures suggesting an 'aging' effect of *in vitro* conditions. IL-1 was a potent inducer of the dopaminergic neuronal phenotype in short-term expanded *in vitro* cultures and was expressed *in vitro* as well as *in vivo* at E14.

Several important conclusions concerning fetal mouse stem cell behavior could be drawn from the results of this work:

Firstly, the results showed for the first time that in fetal mouse mesencephalic NPCs dopaminergic neurons differentiate from precursors without cell division, therefore consuming those progenitors. Therein fetal mouse NPCs differ significantly from rat and human NPCs or respond differently to the same *in vitro* conditions that need to be optimized for fetal mouse NPCs.

Secondly, less committed precursors find appropriate conditions to proliferate but not to generate the more committed DA precursors that are able to generate dopaminergic neurons.

The hallmarks of stem cells, self-renewal and multipotentiality, seem to be part of a delicate balance, that, when unsettled, goes in favor of one side without the possibility of returning to the previous status.

Further research should focus on two coherent issues: the isolation of more pure populations of progenitors and the more precise characterization of progenitor populations to find out which *in vitro* conditions need to be provided to keep the balance between proliferation and differentiation potential. The knowledge gained about stem cells this way would help establish cell sources for transplantation strategies.

1. Introduction

1.1 Stem cells in regenerative medicine

Stem cells are an undifferentiated cell type and are able to divide continuously and produce cells that are committed towards a lineage of mature cells that become functionally integrated into an organism. These two hallmarks of stem cells are called self-renewal and differentiation capacity.

Stem cells are classified according to their potential to give rise to a larger or smaller variety of committed cells. Totipotent stem cells are able to give rise to a new individual if provided with appropriate maternal support. Thus only the zygote and its immediate progeny up to the blastula stage are totipotent cells, because each individual cell can newly generate all embryonic and extra-embryonic tissues required for mammalian development (17). These cells are termed embryonic stem cells (ES cells). The cells of the Inner Cell Mass (ICM) are pluripotent because they can give rise to all cell types of the organism. However, in contrast to totipotent cells they can only reintegrate into a developing organism, they are not able to form a whole organism alone. Stem cells that are committed to differentiate into all cell types of one germ layer have been termed multipotent stem cells, such as neural stem cells. Unipotent cells are restricted to giving rise to only one cell lineage and cell type, such as B-lymphocytes.

Because of their developmentally inhabited potential, stem cells hold a large therapeutic promise. The underlying reasons in many diseases are the malfunction or death of a more or less specific cell type or confined area(s) within the organism. An ideal treatment would therefore be to substitute diseased cells with the healthy counterpart.

This has long been successful for Leukemia, which is a cancer of leukocytes (white blood cells). Like other blood cells, leukocytes are made in the bone marrow through a process that begins with multipotent adult stem cells. Leukemia is the result when leukocytes begin to grow and function abnormally, impeding immunological functions and functions of other organs. Routine treatment for leukemia is chemotherapy, which uses potent

drugs to target and kill the abnormal cells. Stem cell transplants from bone marrow, peripheral blood or umbilical cord blood stem cells are then used to rebuild the bone marrow.

There are a number of neurodegenerative diseases where stem cells could prove a good treatment but have not been successfully employed:

Stroke causes an irreversible, locally restricted loss of neuronal circuitry and supporting glial cells with consecutive functional deficits and disabilities. Preclinical and clinical cell-based studies focus on stem cells derived from the adult central nervous and hematopoietic systems. An increasing number of studies provide evidence that hematopoietic stem cells, either after stimulation of endogenous stem cell pools or after exogenous hematopoietic stem cell application, improve functional outcome after ischemic lesions (reviewed by (57,187)).

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating disease that affects the central nervous system (CNS). In a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), injection of neural stem cells into the tail vein resulted in an increase in glial cells (135).

Amyotrophic lateral sclerosis (ALS) is a progressive disease that manifests as a gradual evolution, spread of weakness and wasting of the affected patient's muscles. The direct underlying cause is the progressive loss of the muscles' motor neurons. Stem cells would have to replace motoneurons and integrate into every segment. Additionally, they needed to make a meter long branch to integrate functionally. Alternatively, the stem cells could be induced to differentiate into supporting cells, glia, or interneurons that might produce factors that would support motor neurons, or perhaps the stem cells themselves might produce such factors (reviewed by (171)). Transplantation studies in a mouse model resulted in a significant increase of the average life span of transgenic mice by 10–20% as well as motor function improvement (38,49). Habisch and colleagues however demonstrated that the cell number might be crucial for a beneficial outcome of a transplantation procedure (58).

Alzheimer's disease (AD) is a neurodegenerative disease that includes progressive cognitive deterioration together with a declining ability to perform activities of daily living, neuropsychiatric symptoms or behavioral

changes. Plaques which contain misfolded peptides called amyloid beta (Aβ) are formed in the brain many years before the clinical signs of Alzheimer's are detected. Immortalized neuroepithelial stem cells injected into various brain regions of 2-years-old rats displayed incorporation and migration in the host brain with substantially reversed cognitive declination associated with aging, suggesting a potential for neuroreplacement strategies also for age-associated neurodegenerative diseases (67). However, the ubiquitous decline of neurons makes it hard to restore all functions by cell replacement strategies.

Parkinson's disease (PD) is characterized by muscle stiffness of limbs and trunk (rigidity), trembling in hands, arms, legs, and face (tremor), impaired balance and coordination (postural instability) and a slowing (bradykinesia) or loss (akinesia) of physical movement. Typical non-motor symptoms include disorders of mood, behavior, thinking, and sensation. The primary symptoms result from a decreased stimulation of the motor cortex by the basal ganglia, caused by the insufficient formation and action of dopamine. Dopamine is produced by dopaminergic neurons in the substantia nigra pars compacta that project to the striatum. Their loss in Parkinson’s disease leads to alterations in the activity of the neural circuits within the basal ganglia that regulate movement.

At present, there is no cure for PD, but medications or surgery can provide relief from the symptoms. L-DOPA (Levodopa), which is considered the gold standard as treatment, is metabolized to dopamine in dopaminergic neurons and compensates the loss of dopamine. Because only 1-5% of L-DOPA reaches the dopaminergic neurons and the rest of the drug is metabolized elsewhere, side-effects occur than can be treated with Carbidopa which prevents premature metabolization of Levodopa. Dopamine agonists and deep brain stimulation are mainly used when Levodopa therapy causes problems or is no longer effective.

Parkinson’s disease is an ideal neurodegenerative disease for transplantation strategies, because studies can focus on a very distinct cell type that dies and cell replacement can focus on a particular area. Additionally, transplanted cells do not need to functionally integrate into the neuronal network of the host; as long as they release dopamine they will

treat the disease symptoms. Many kinds of cells have been considered for cell replacement therapies in Parkinson's disease (reviewed by (64,171)).

Primary cells have been successfully transplanted to diseased brains (44,86,99,125) but they impose ethical as well as logistical problems because they are taken from aborted fetuses and 8-9 fetuses are needed to isolate a cell number that will treat one patient (98,99).

Stem cells promise to overcome this shortage (reviewed by (47,62,70,116,162)). ES cells have the largest potential because they self-renew indefinitely and generate virtually all cell types of an organism. However, until now it has been impossible to suppress the potential of ES cells for uncontrolled growth leading to tumors in transplanted hosts (16,19,33,100). Neural stem cells (NSCs) are able to differentiate into the major cell types of the nervous system and have been shown to integrate and improve motor function in mouse models of PD (18,24,43,179). They are not prone to uncontrolled cell proliferation in the host, but the yield of expansion which is crucial for cell numbers for transplantation is lower than with ES cells.

In summary, the ideal cell source for transplantation strategies would be stem cells that are expanding fast and virtually forever, retaining their differentiation capacity and inhabiting no risk to form tumors. Their production would be possible to standardize. Neural stem/progenitor cells (NPCs) from fetal tissue are a rapidly dividing source of cells that are able to generate cells of all three neural lineages and do not divide uncontrolled once transplanted into a host organism. Thus, at present, they are the ideal cell source for cell replacement strategies.

1.2 Neural stem/progenitor cells

Stem cells that are multipotent and have the potential to generate all cells from the nervous tissue are tissue-specific neural stem cells. Because they divide for a long time *in vitro,* yet probably not unlimited, they are usually referred to as neural progenitor cells (NPCs)(12).

They were first detected in 1965 by Altman et al. (3,4) but were then long forgotten until the 1990s when their existence was confirmed by several researchers in multiple brain areas in the adult and fetal brain (2,23,39,53,103,175). Accordingly, clonogenic NPCs can be directly isolated from fetal or adult nervous tissue, or derived from ES cells (29,47,78, 101,116,168,178) (**Fig. 1**).

NPCs have been categorized as multipotent stem cells derived from the nervous system with the capacity to regenerate and to give rise to cells belonging to all three major cell lineages of the nervous system, namely neurons, oligodendrocytes and astrocytes (1,8,29,47,78,101,116,136, 168,178).

In vivo, fetal neural stem cells form from neuroectodermal tissue when neurogenesis starts. The unilayered neuroepithelial tissue transforms into tissue with multiple cell layers between embryonic day (E) 10 and E12 in most areas of the mouse brain (59,123). Neuroepithelial cells give rise to radial glial cells that appear to be more fate-restricted and successively replace neuroepithelial cells. In contrast to neuroepithelial cells, radial glial cells show astroglial markers, such as GLAST (astrocyte-specific glutamate transporter), S100β, Gfap (glial fibrillary acidic protein), vimentin and BLBP (brain-lipid-binding protein)(59,110,123). Otherwise both stem/progenitor cell types are related. It has been proposed that they first divide symmetrically to increase the pool of stem cells, but later divide asymmetrically, generating a stem cell that remains in the ventricular zone and a daughter cell that becomes either a more restricted (intermediate) progenitor or terminally differentiates and migrates radially outward (52,60). The accumulation of daughter cells thickens the developing brain and radially stretches neuroepithelial cells. This division in the subventricular zone amplifies the number of cells produced by a given NPC division and may be an important determinant of brain size, since species with larger brains have a larger pool of intermediate progenitors (112).

In vitro, most studies regarding fetal midbrain tissue involved rodent embryos at embryonic day (E)14 to E15 (101,136,158,159), while on human midbrain material, samples of 6-9 weeks post-fertilization (88,160) were

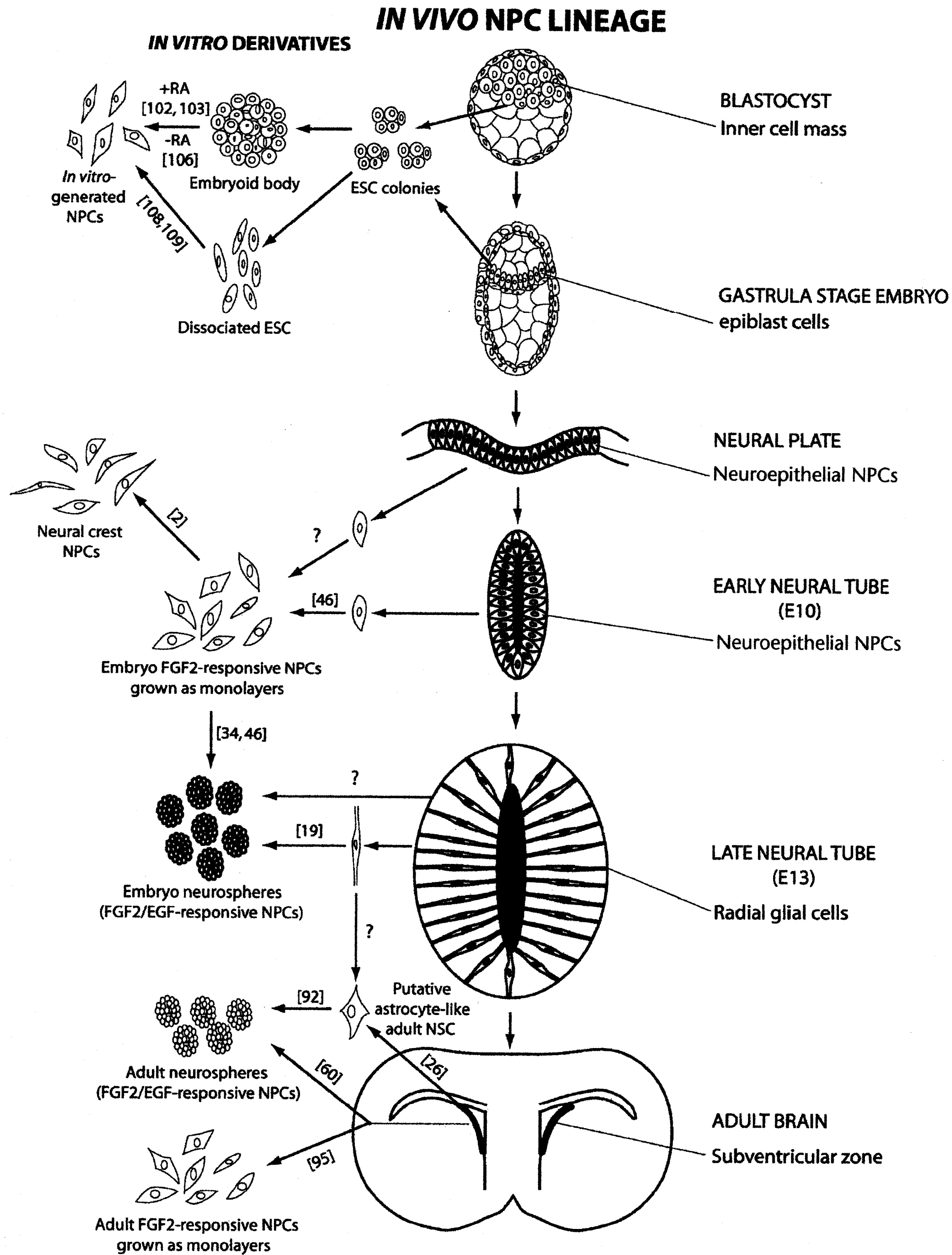


Fig. 1 The origin of NPCs and *in vitro* derivatives. Different populations of NPCs appear during embryo development and in the adult brain (center of scheme). During neural plate induction and soon after neural tube closure, the majority of cells are neuroepithelial NPCs. As neural tube development proceeds, radial glial cells appear and become an important NPC population. Eventually, radial glial cells could generate the putative astrocyte-like NSC present in the adult subventricular zone. Different culture techniques allow *in vitro* expansion of NPCs (left of scheme). Multipotent FGF2-responsive NPCs can be expanded *in vitro* under adherent conditions from neural tubes at very early developmental stages. Embryo FGF2-responsive NPCs generate *in vitro* the Fgf-2/Egf-responsive, neurosphere-forming NPCs. Radial glial cells and probably other precursors naturally become Fgf-2/Egf-responsive, neurosphereforming NPCs during neural tube development. The lineage relationship between embryo and adult NPCs and these relationships with their *in vitro* derivatives remains uncertain ("?" in the scheme). Finally, NPCs can be completely generated *in vitro* from ES cells, pluripotent stem cells derived from the blastocyst inner cell mass (top of the scheme). ES cells differentiate into NPCs *in vitro* through formation of embryoid bodies or as adherent monolayers from dissociated cells. According to Baizabal et al. (12).

used (reviewed by (161)). After isolation, NPCs were plated in low-attachment culture flasks in serum-free media containing serum supplements, such as N2 or B27 (144,159,160,178). Gensburger and co-workers described fibroblast growth factor-2 (FGF-2; formerly basic fibroblast growth factor) inducing proliferation of neural precursors in embryonic hippocampal cultures (50). Later epidermal growth factor (Egf) was found to stimulate the growth of striatal precursors retaining their ability to differentiate into all major types of CNS cell types (140). Since these early studies most protocols for *in vitro* proliferation of both fetal and adult NPCs use the mitogens Fgf-2 and Egf alone or in combination in serum-free-media ((158), for review see Svendsen et al. (168)). Various other epigenetic approaches have been employed for enhanced *in vitro* cultivation of NPCs including erythropoietin (156) and ATP (97). For some of these proliferating factors it is not completely clear how these factors influence the differentiation potential into neurons and glial cells (185).

Another established neuroproliferative substance is leukemia inhibitory factor (LIF): When added to the culture medium, LIF allows continuous cell growth in human NPC cultures (25). LIF acts through the gp130 signal transducing subunit and is also required for the continuous growth of mouse, but not human ES cells. LIF appears to maintain these cultures in a proliferative state by preventing differentiation. Another strategy to enhance proliferation and to increase culture time is genetic manipulation by transduction of immortalizing genes into neural progenitors (for review, see (113,185)).

Fetal stem or progenitor cells grow in cellular aggregates called “neurospheres”, but alternatively they can be expanded as adherent cell cultures on poly-L-ornithin/fibronectin-coated surfaces with both Egf and Fgf-2 as mitogens.

Crucial for many types of neural stem (progenitor) cell cultures seems to be reduced atmospheric oxygen (159,160,163). For example, in contrast to precursor cells derived from human forebrain samples, which are easily expanded by using Egf/Fgf-2 in 21% O2, precursors derived from the human mesencephalon do not show any proliferation under these conditions or do not differentiate into dopaminergic neurons *in vitro* (160,169,170). Under

these conditions, Egf/Fgf-2-responding NPCs with dopaminergic potential from rat and human midbrain tissue of less than 20 weeks post fertilization can be successfully expanded for over 1 year. However, the way in which these proliferation factors influence the differentiation of NSCs into distinct subsets of neurons or glial cells is currently not completely clear (185). Smith et al. showed that there are fundamental differences between NPCs of different species. In their study, rat NPCs proliferated more rapidly than mouse NPCs, but the authors also stressed that they used recombinant human growth factors which might have a higher affinity to rat receptors (158). Fetal rodent (122,166,167) as well as human (45,169) NPCs have been successfully transplanted into animal models of Parkinson's disease and survive, differentiate and migrate in the host brain, providing proof of principle for stem cell transplantations. However, optimal conditions resulting in better symptomatic relief or recovery have not been specified to date.

Adult neural stem cells reside in the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus. Studies demonstrated the isolation and *in vitro* expansion of neural progenitor cells (NPCs) from different regions of the adult rodent brain including cortex, amygdala, hippocampus and SVZ (3,4,8,14,72,88,93,104,106,118,119, 124,146,184); reviewed by (48,127,174), but there are only a few reports about adult human NPCs *in vitro* (63,72,88,108,146). Adult NPCs have been transplanted with varying outcome (30,120,143).

Derivation from ES cells yields neural stem cells with a large potential (69,173) that can subsequently be efficiently differentiated into neurons (75,76,91). However, their proliferative potential seems to be responsible for the high risk of tumor formation after transplantation (5,16). In addition, there are immense ethical concerns regarding the use of human ES cells and government restrictions that will, at least for the forthcoming years, render it unlikely that these cells will be therapeutically employed in many countries.

Furthermore, there is evidence for multipotent adult stem cells from other tissues that seem to have the potential to transdifferentiate into neuroectodermal lineages (71,148,176). In contrast to ES cells, multipotent stem cells are often defined by the organ from which they are derived. Multipotent adult stem cells are of special interest with regard to autologous

transplantation approaches because immunological rejection could be circumvented. An important source of multipotent adult stem cells is bone marrow stromal cells (MSCs); also known as mesodermal stromal cells or mesenchymal stem cells. In contrast to human ES cells or human brain-derived NPCs, human MSCs (hMSCs) are easily isolated and can be expanded over a long period of time without serious ethical and technical problems. MSCs can be cultivated *in vitro* and contain progenitors capable of generating osteocytes, chondrocytes and adipocytes (46,139,153). Several reports demonstrate that under specific experimental conditions MSCs can also differentiate into cells that are not part of their normal repertoire: skeletal and cardiac muscle, hepatocytes, and glia (11,41,61,109,126,148,149). Functional neurons however have been conclusively demonstrated only in mouse and genetically modified human MSCs.

Various molecular markers identifying NPCs and their sequential differentiation stages are known to date (48). The first marker defining NPCs was the intermediate filament protein nestin (92), but some neural precursor cells are nestin-negative (89) and, on the other hand, nestin is expressed by other cell types, including non-neural cells (154). Other stem cell markers include Musashi1 and 2, Prominin-1 (cd-133), Notch1, Sox-1, and Sox-2 (36,42,47,74,85,90,150,177,182). However, it is necessary to point out that these markers are arbitrary and their significance depends in part on the state and microenvironment in which the cells are located or isolated from.

Thus, NPCs are usually identified operationally by their biological behavior after isolation: They grow in floating, multicellular aggregates, so-called ‘neurospheres’ (47,116) and are able to self-renew and differentiate into the three major cell types of the nervous tissue. Uchida and colleagues performed an extensive analysis of surface markers on neurospheres from human origin. They defined a subset of human NPCs as phenotypically CD133-positive, but negative for the hematopoietic stem cell marker CD34 and negative for CD45 (177). Recently the expression of various proneural genes like *SOX1*, *Musashi1*, *Otx-1*, *Otx-2*, *NeuroD1* and *Neurogenin-2* was reported in mammalian NPCs, showing their neuroectodermal origin (9,22).

NPCs have been isolated from different species and origins with different characteristics and potentials but so far no source produced stem cell cultures that behaved optimal in transplantation studies in terms of graft survival, differentiation and tumor growth. Therefore, the characteristics of NPCs and their manipulation need to be investigated more detailed.

1.3 Generation of dopaminergic neurons

In vivo mesencephalic dopamine (mDA) neurons are generated in the ventral midbrain at three different positions (substantia nigra (A9), ventral tegmental area (A10) and the retrorubral field (A8)) during a restricted time period starting at embryonic day (E) 10.5 in the mouse (13). This is accomplished by a complex interplay of different instructive signals from several important signaling centers in the brain. Along the dorsoventral axis, the ventral (floor plate) and the dorsal (roof plate) midline are responsible for the secretion of signaling molecules, like the lipid-modified glycoprotein Sonic hedgehog (Shh) from the ventral midbrain floorplate. Midbrain patterning along the anterior-posterior axis is achieved mainly by the midbrain-hindbrain boundary (MHB), at or across which Wnt1, Fgf-8, Pax2/5 and Engrailed (En) 1 and 2 are secreted. It was shown that Shh and Fgf-8 together were necessary (and sufficient) to generate ectopic mDA neurons in rat embryo cultures (186). Work with knockout mice and mouse embryo explant cultures described that additionally Wnt1 is needed for the induction of mDA neurons (137). Downstream of Shh, Fgf-8 and Wnt1, Lmx1b, Lmx1a and further Pitx3 and Nr4a2 (Nurr1) are required for the normal generation of dopaminergic neurons (6,137,147,157).

In vitro adult stem (progenitor) cells (aNPCs) from different neurogenic and non-neurogenic regions have the potential to give rise to cells belonging to all three major cell lineages of the nervous system; neurons, oligodendrocytes, and astrocytes *in vitro* (95,129,131,155). Hermann and colleagues differentiated NPCs from the mouse midbrain and investigated their dopaminergic differentiation potential. They established the isolation, expansion and *in vitro* characterization of adult mouse midbrain

NSCs and their differentiation into functional nerve cells including dopaminergic neurons (65).

Fetal NPCs have also been shown to generate oligodendrocytes, neurons, and astrocytes *in vitro* (1,8,29,78,101,136,168,178) in a ratio of approximately 1:5:25 (56,73) after the removal of growth factors in serum-free media on standard culture growth surfaces, such as poly-L-lysine or laminin (55).

In the CNS, patterning in the anterior-posterior and dorsal-ventral axes occurs early, concomitantly with neural induction. Indeed, neurospheres generated from different CNS regions express region-appropriate markers and generate region-appropriate progeny. Spinal cord stem cells generate spinal cord progeny (115), whereas stem cells from the forebrain generate more gamma-aminobutyric-acid- (GABA-) containing neurons than dorsal stem cells (136). Consistently, only NPCs isolated from the midbrain differentiate into functional mature dopaminergic neurons (101,136,150,160). Consequently, it has been postulated that stem cells and restricted precursors exhibit regionalization (66,79,128,158) but may not be committed to that fate (66). Additionally, Smith et al. showed that the potential of rat NPCs to generate neurons rapidly disappears *in vitro* under the culture conditions used (Egf,Fgf-2, atmospheric oxygen levels (158)).

This differentiation programme can be influenced *in vitro* by addition of growth factors or cytokines. Since NPCs have been suggested as a new source of tissue for regenerative therapy in PD (15,35), several studies on midbrain-derived NPCs have focused on the induction of a dopaminergic phenotype. During the last few years many aspects of the genetic and epigenetic control of the induction and phenotypic maturation of dopaminergic neurons have been characterized (for review see (133). Instructive signals for the generation of progenitors capable of differentiation into dopaminergic neurons are Fgf-8, Shh and Wnt1 (**Fig. 2**). Midbrain derived precursors apparently do not require those signals (178) but need activation of signaling cascades leading to the expression of two crucially involved transcription factors: Nr4a2 and Pitx3 (**Fig. 2**).

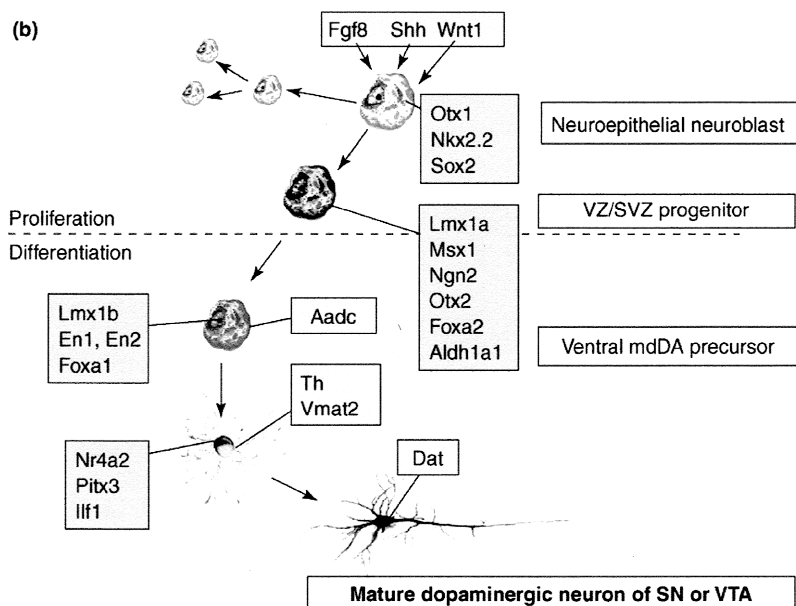


Fig. 2 Downstream of the inductive signals Fgf8, Shh and Wnt1, expression of transcription factor genes and mdDA-specific genes is acquired at different stages of differentiation: initially, the expression of the Otx1, Nkx2.2 and Sox2 genes, followed by Lmx1a, Msx1, Ngn2, Otx2 and Foxa2 transcription factors. As part of the transcriptional code, several of these are repressed during further development (e.g. Sox2, Nkx2.2 and Nkx6.2). The early stage of mdDA-specific differentiation (at E9–E10 in mice) further involves Lmx1b, the engrailed factors En1 and En2, and Foxa1. At this stage, the first gene for dopamine synthesis, amino acid decarboxylase (Aadc), is induced. Subsequently, transcription factors for terminal differentiation are induced: Nr4a2 (Nurr1) at E10.5 and Pitx3 at E11.5. Pitx3 is required for survival of some terminally differentiating subsets of mdDA neurons. Ilf1 is a newly discovered forkhead factor, expressed at E12.5. Nurr2 is required for induction of tyrosine hydroxylase (Th) at E11.5, of vesicular monoamine transporter 2 (Vmat2) at E12.5, and of the dopamine transporter (Dat) at E14, at which point the cells are mature mdDA neurons of the substantia nigra (SN) or ventral tegmental area (VTA). Aldehyde dehydrogenase A1 (Aldh1a1) is expressed in proliferating mdDA progenitors. In this figure, expression of transcription factors is linked to stages of mdDA development as marked by components of dopaminergic-neuron synthesis and transmission, but no functional genetic relationship is inferred. According to Burbach and Smidt (20).

For example, Nr4a2 was reported to be upregulated by the differentiation factor interleukin-1 (IL-1) in mesencephalic precursor cells from rat (26). Also, the overexpression of Nr4a2 induced a midbrain dopaminergic phenotype after the stimulation of NPCs with astrocyte-conditioned medium (81,82,183). Consistently, Ling and co-workers demonstrated with rat-derived neural progenitor cells that IL-1 was able to induce the dopaminergic phenotype with a significantly increased effect by adding other factors, such as IL-11, LIF, glial-derived neurotrophic factor (GDNF) and membrane fragments in midbrain NPCs, but not in striatal NPCs (101). These dopaminergic cells show all the major morphological features and functional properties of mature dopaminergic cells, such as dopamine production/release and the expression of sodium and

hyperpolarization induced cation channels (159). IL-1 was potent in inducing the dopaminergic phenotype not only in rat but also in human NPCs (26,74,101,160). Transforming growth factor β was used to increase dopaminergic cell numbers in short-term fetal mouse NPC culture (10d), whereas double knockout mice (Tgf-β2 $^{-/-}$/Tgf- β3 $^{-/-}$) displayed significantly less Th+ cells in the midbrain than wt mice, showing that Tgf β is a beneficial or necessary factor for dopaminergic neurons (145). Wnt5a overexpression and recombinant protein was used to obtain more TH+ cells in rat NPCs (27,28,132). Transplantation of Wnt5a overexpressing cells resulted in significant cellular and functional recovery in parkinsonian mice. When Nr4a2 and Ngn2 were overexpressed in ventral mesencephalic NPCs from E11.5 mice, dopaminergic neurons with mature phenotypes were induced (7,183). Other ways have not been successful in generating dopaminergic neurons from long-term expanded fetal mouse NPCs (31,83). Long-term expansion however is essential to stem cell research, because it is necessary to obtain large quantities of cells for future cell replacement strategies and to eliminate primary cells carried over from the isolation process.

All reports using fetal mouse NPCs for the establishment of substances promoting dopaminergic or neuronal differentiation have used short-term expanded cultures, making it difficult to ensure that real differentiation instead of dopaminergic neuron survival took place (28,40,132,145,150). In contrast, several reports demonstrated long-term expansion and subsequent dopaminergic differentiation of human and rat fetal NPCs (26,27,84,100,117,159,160,168).

Transplantation of expanded and *in vitro* differentiated neural precursors resulted in a survival rate of 3-5% of the cells and behavioral recovery of parkinsonian rats with no indication of uncontrolled growth of the transplanted cells (164) making NPCs from fetal tissue to date the only suitable cell source for transplantation strategies in neurodegenerative diseases because they do not form tumors and have the potential to generate functional dopaminergic neurons.

Additionally, Parkinson's disease is the ideal neurodegenerative disease for transplantation strategies because of a confined cell loss. To

approach the goal of cell replacement the precise regimens governing the differentiation of fetal NPCs into dopaminergic neurons need to be elucidated.

1.4 Aims of the thesis

Fetal mouse NPCs are a crucial tool for investigations on neural stem cell differentiation, because they make the employment of transgenic animals *in vivo* and cells *in vitro* possible. So far only short-term expanded NPC cultures displayed dopaminergic neurons and it is not clear whether this was due to differentiation or a result of increased survival of primary dopaminergic neurons.

The aims of the thesis were to characterize mouse fetal NPCs, to establish the long-term expansion of fetal mouse NPCs and the generation of dopaminergic neurons in long-term expanded fetal mouse NPCs, to investigate the signaling mechanisms involved in the differentiation of mouse fetal NPCs towards the dopaminergic phenotype and to compare NPCs that were cultivated for short periods *in vitro* with long-term expanded NPCs.

To this end fetal NPCs had to be isolated and long-term expanded. This was necessary to obtain large quantities of cells and to overcome contaminations of primary cells from the isolation process. The proliferation as well as differentiation potential of NPCs from different developmental origins (E10, E12 and E14) was described. Their ability to develop neural cells of all three lineages as well as neuronal subtypes was determined. The activation of p38 and Erk1/2 signaling cascades in response to the initiation of dopaminergic differentiation by potent inducers of the dopaminergic phenotype was analyzed. The expression of signaling molecules potentially involved in the dopaminergic specification was investigated. Additionally, short-term expanded NPCs were analyzed for their proliferation and differentiation potential. They were FACS-sorted to prove the *in vitro* differentiation from precursor cells. Finally, short-term and long-term NPC cultures were compared on the mRNA level to find markers describing stem cells *in vitro*.

The results to these experiments should help answer the following questions: Can dopaminergic neurons be generated *de novo* from fetal mouse NPCs *in vitro*? Which signaling pathways/molecules are involved in the dopaminergic specification of fetal mouse NPCs? Do short-term and long-term NPCs differ from each other?

The results of this project will have an impact on neural stem cell science because no real differentiation of fetal mouse NPCs has been demonstrated to date.

2. Material

2.1. Instruments

Instrument	Designation	Company
Balance	SBA 52	Scaltec (Göttingen, Germany)
Cell incubator	Hera Cell 150	Heidolph (Düsseldorf, Germany)
Cell Prep Instruments	various	VWR (Darmstadt, Germany)
Centrifuge, big	5810 R	Eppendorf (Hamburg, Germany)
Centrifuge, small	Mini Spin Plus	Eppendorf (Hamburg, Germany)
Clean bench	Hera Safe	Heraeus (Berlin, Germany)
Confocal Microscope	DMIRE2	Leica (Braunschweig, Germany)
Cryostat	2800 FrigoCut E	Reichert-Jung (Nussloch, Germany)
FACS Sorter	FACSCalibur	BD Biosciences (Franklin, USA)
Fluorescence microscope	DM IRE2	Leica (Braunschweig, Germany)
Magnetic stirrer	RCT basic	IKA Labortechnik (Staufen, Germany)
Orbital shaker	Polymax 1040	Heidolph (Düsseldorf, Germany)
Perfusion Pump	Dynamax	Rainin (Oakland, USA)
PCR cycler	MX 3000 P	Stratagene (La Jolla, USA)
Photometer	Bio Photometer	Eppendorf (Hamburg, Germany)
Plate reader	Sunrise	Tecan (Crailsheim, Germany)
Rotator	OV 3	Biometra (Göttingen, Germany)
Thermocycler	Thermomixer Comfort	Eppendorf (Hamburg, Germany)
Vortexer	L 46	GLW (Würzburg, Germany)
Water bath	SW 22	Julabo (Seelbach, Germany)

2.2. Chemicals and Consumables

Substance or Consumable	**Company**
Agarose	Biozym LE (H. Oldendorf, Germany)
BrdU (5'Bromo-2'-Desoxyuridine)	Serva (Heidelberg, Germany)
Cell culture filter	Millipore (Billerica, USA)
Eight-well chambers	BD Biosciences (Franklin, USA)
Fibronectin	Sigma-Aldrich (St. Louis, USA)
Glucose	Sigma-Aldrich (St. Louis, USA)
Microscope slides	Langenbrinck (Teningen, Germany)
Microscope cover slips	Marienfeld (Germany)
Non-adhesive culture flasks	Greiner bio-one (Frickenhausen, Germany)
Paraformaldehyde (PFA)	Sigma-Aldrich (St. Louis, USA)
Poly-D-Lysine	Sigma-Aldrich (St. Louis, USA)
Poly-L-Ornithine	Sigma-Aldrich (St. Louis, USA)
Sucrose	VWR (Darmstadt, Germany)
Tris	Roth (Karlsruhe, Germany)
Triton-X 100	Serva (Heidelberg, Germany)
Trypsin (cell-culture)	Gibco (Invitrogen; Carlsbad, USA)
Trypsin (cell prep)	Sigma-Aldrich (St. Louis, USA)

General consumables and chemicals were purchased from Sigma, Roth, Merck, Greiner or Eppendorf.

2.3. Solutions, Buffers

Substance or Consumable	Company
5x Taq M Enhancer	Eppendorf (Hamburg, Germany)
10x Taq Buffer	Eppendorf (Hamburg, Germany)
Taq Polymerase	Eppendorf (Hamburg, Germany)
Accustain	Sigma-Aldrich (St. Louis, USA)
dNTP's (2mM)	Eppendorf (Hamburg, Germany)
Donkey serum	Jackson Immunoresearch (USA)
HBSS	Gibco (Invitrogen, Carlsbad, USA)
Hoechst 33342	Molecular Probes (Eugene, USA)
PBS	Gibco (Invitrogen, Carlsbad, USA)
Tissue Tek O.C.T. Compound	Jung (Nussloch, Germany)
Vectashield	Vector (Burlingame, USA)
Ultra Pure (PCR-) Water	Roth (Karlsruhe, Germany)

2.4. Kits

Kit	Company
Brilliant® SYBR® Green QRT-PCR Master Mix	Stratagene (La Jolla, USA)
RNeasy® Mini	Qiagen (Hilden, Germany)
DNeasy Blood and Tissue	Qiagen (Hilden, Germany)

2.5. Enzymes, Growth factors and Antibodies

2.5.1. Enzymes

Enzyme	Company
DNase (for Cell Prep)	Sigma-Aldrich (St. Louis, USA)
DNase (for RNA isolation)	Qiagen (Hilden, Germany)
Trypsin	Sigma-Aldrich (St. Louis, USA)

2.5.2. Growth factors

Growth factor	Company
Egf (human, recombinant)	Invitrogen (Carlsbad, USA)
Fgf-2 (bFGF) (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
IL-1 (Interleukin-1) (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
Forskolin (human, recombinant)	Sigma-Aldrich (St. Louis, USA)
Fgf-8 (human, recombinant)	R&D Systems (Minneapolis, USA)
Shh (human, recombinant)	R&D Systems (Minneapolis, USA)

2.5.3. Primary Antibodies

Antibody	Company
Mouse anti-Nestin (1:500)	Chemicon (Billerica, USA)
Mouse anti-Galactocerebrosidase (GalC; 1:750)	Chemicon (Billerica, USA)

Mouse anti-Microtubuli-associated Protein (Map2ab) 2ab(1:500)	BD Biosciences (Franklin, USA)
Rabbit anti-ß-III-Tubulin (1:2000)	Covance (Princeton, USA)
Rabbit anti-tyrosine hydroxylase (Th; 1:200)	PelFreeze (Rogers, USA)
Rabbit anti-Nestin (1:500)	Abcam (Cambridge, UK)
Rabbit anti-Ki-67 (1:500)	Novocastra (Newcastle, UK)
Rabbit anti GABA (1:1000)	Sigma (St. Louis, USA)
Rabbit anti Serotonin (1:1000)	Sigma (St. Louis, USA)
Rabbit anti Glutamate (1:2000)	Sigma (St. Louis, USA)
Rabbit anti Chat (1:1000)	Chemicon (Billerica, USA)
Rat anti-5'Bromo-2' desoxyuridine (BrdU;1:100)	Abcam (Cambridge, UK)
Chicken anti Glial Fibrillary Acetic Protein (Gfap; 1:1000)	Abcam (Cambridge, UK)

2.5.4. Secondary Antibodies

Antibody	**Company**
Alexa Fluor® 647 conjugated donkey anti-goat antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 647 conjugated donkey anti-mouse antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 488 conjugated donkey anti-mouse antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 555 conjugated donkey anti-mouse antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 488 conjugated donkey anti-rabbit antibody	Invitrogen-Molecular Probes (Carlsbad, USA)

Alexa Fluor® 594 conjugated donkey anti-rabbit antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 647 conjugated donkey anti-rabbit antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Alexa Fluor® 555 conjugated donkey anti-rat antibody	Invitrogen-Molecular Probes (Carlsbad, USA)
Cy5 conjugated donkey anti chick antibody	Dianova (Hamburg, Germany)
Cy3 conjugated donkey anti chick antibody	Dianova (Hamburg, Germany)
FITC conjugated donkey anti chick antibody	Dianova (Hamburg, Germany)

2.6. Oligonucleotides

Name	5'-3' sequence	NBCI ACC #	Product length
mm_HMBS_fwd **mm_HMBS_rev**	TGTATGCTGTGGGTCAGGGAG CTCCTTCCAGGTGCCTCAGA	NM_013551	144 bp
mm_Aldh1a1_fwd **mm_Aldh1a1_rev**	TATGTTCTTGGAAATCCTCTGAC GCTCCTTCTTTCTTCCCACTC	NM_013467.3	110 bp
mm_Egf-R _fwd **mm_Egf-R _rev**	AGGGGACCAGACAACTGCAT GCATCTGCATACTTCCAGACC (2 transcript variants)	NM_207655 NM_007912	119 bp
mm_En1_fwd **mm_En1_rev**	ACACAACCCTGCGATCCTAC GTGCGTGGACCAGAGGAC	NM_010133.1	138 bp
mm_En2_fwd **mm_En2_rev**	GACTCGGACAGCTCTCAAGC ACCTGGGACCTGAAGAAGG	NM_010134.3	109 bp
mm_FoxA1_fwd **mm_FoxA1_rev**	CAGGAGGCCTACTCCTCTGTC GCTCGTGGTCATGGTGTTC	NM_008259	102 bp
mm_FoxA2_fwd **mm_FoxA2_rev**	GCCAGCGAGTTAAAGTATGC TCATGTTGCTCACGGAAGAG	NM_010446	110 bp
mm_GFAP_fwd **mm_GFAP_rev**	GATCTATGAGGAGGAAGTTCG TCTGCAAACTTAGACCGATACC	NM_010277.3	183 bp
mmGfp_fwd **mmGfp_rev**	AAGTTCATCTGCACCACCG TGCTCAGGTAGTGGTTGTCG (amplifies genomic DNA)	n/a	~450 bp

mm_Lmx1a_fwd **mm_Lmx1a_rev**	TGCAGAAAGGTGAGGGAGAC TGTTCTGTTGGTCCTGTTGC	NM_033652	139 bp
mm_Lmx1b_fwd **mm_Lmx1b_rev**	GGCCAAGAGGTTCTGTCAAG AAGGGGTCGCTACTTCCGTA	n/a	
mm_Map2ab_fwd **mm_Map2ab_rev**	AAAGTCACTGATGGAATAAGC CCAGGTCAGAACCAATTCGCA	NM_008632.2	172 bp
mm_MBP_fwd **mm_MBP_rev**	CACGGGCATCCTTGACTC GCCGTGCTGCGACTTC	n/a	
mm_Msi_fwd **mm_Msi_rev**	GCAGACCACGCAGGAAG CGCCAGCACTTTATCCAC	NM_008629.1	151 bp
mm_Msx1_fwd **mm_Msx1_rev**	CAGCCAGACGGCTGAGTC TACTGCTTCTGGCGGAACTT	NM_010835	124 bp
mm_Nes_fwd **mm_Nes_rev**	TGGAACAGAGATTGGAAGGC TCTTGAAGGTGTGCCAGTTGC	n/a	
mm_Ngn2_fwd **mm_Ngn2_rev**	CGGCGTCATCCTCCAAC CGGGTAGAGGACGAGAGAGG	NM_009718.2	179 bp
mm_Nkx2.2_fwd **mm_Nkx2.2_rev**	GCCTCCAATACTCCCTGCAC TTGTCATTGTCCGGTGACTC	NM_010919	103 bp
mm_Nurr1_fwd **mm_nurr1_rev**	TGTCAGCACTACGGTGTTCG AGGGTAAACGACCTCTCCG	NM_013613.1	226 bp
mm_Otx1_fwd **mm_Otx1_rev**	GTTCACAGCTGGACGTGCT AACCAGACCTGGACTCTGGA	NM_011023	118 bp
mm_Otx2_fwd **mm_Otx2_rev**	GGTATGGACTTGCTGCATCC GCTGTGCCCTAGTAAATGTCG	NM_144841	112 bp
mm_Pitx3_fwd **mm_Pitx3_rev**	GTGGACTCCTACAGATTGGC CGGATGTGACAGGATTCG	n/a	
mm_Prom_fwd **mm_Prom_rev**	ACCAACACCAAGAACAAGGC GGAGCTGACTTGAATTGAGG	NM_008935.1	349 bp
mm_Sox-2_fwd **mm_Sox-2_rev**	GAACGCCTTCATGGTATGGT TTCTCGGTCTCGGACAAAAG	NM_011443	126 bp
mm_Th_fwd **mm_Th_rev**	GCCTTCCGTGTGTTTCAGTG GGCAAATGTGCGGTCAGC	NM_009377.1	126 bp
mm_Tuj1_fwd **mm_Tuj1_rev**	TGAGGCCTCCTCTCACAAGT TAAAGTTGTCGGGCCTGAAT	NM_023279	116 bp

2.7. Cell Culture Media

Expansion medium (EM) I (Serum-free)

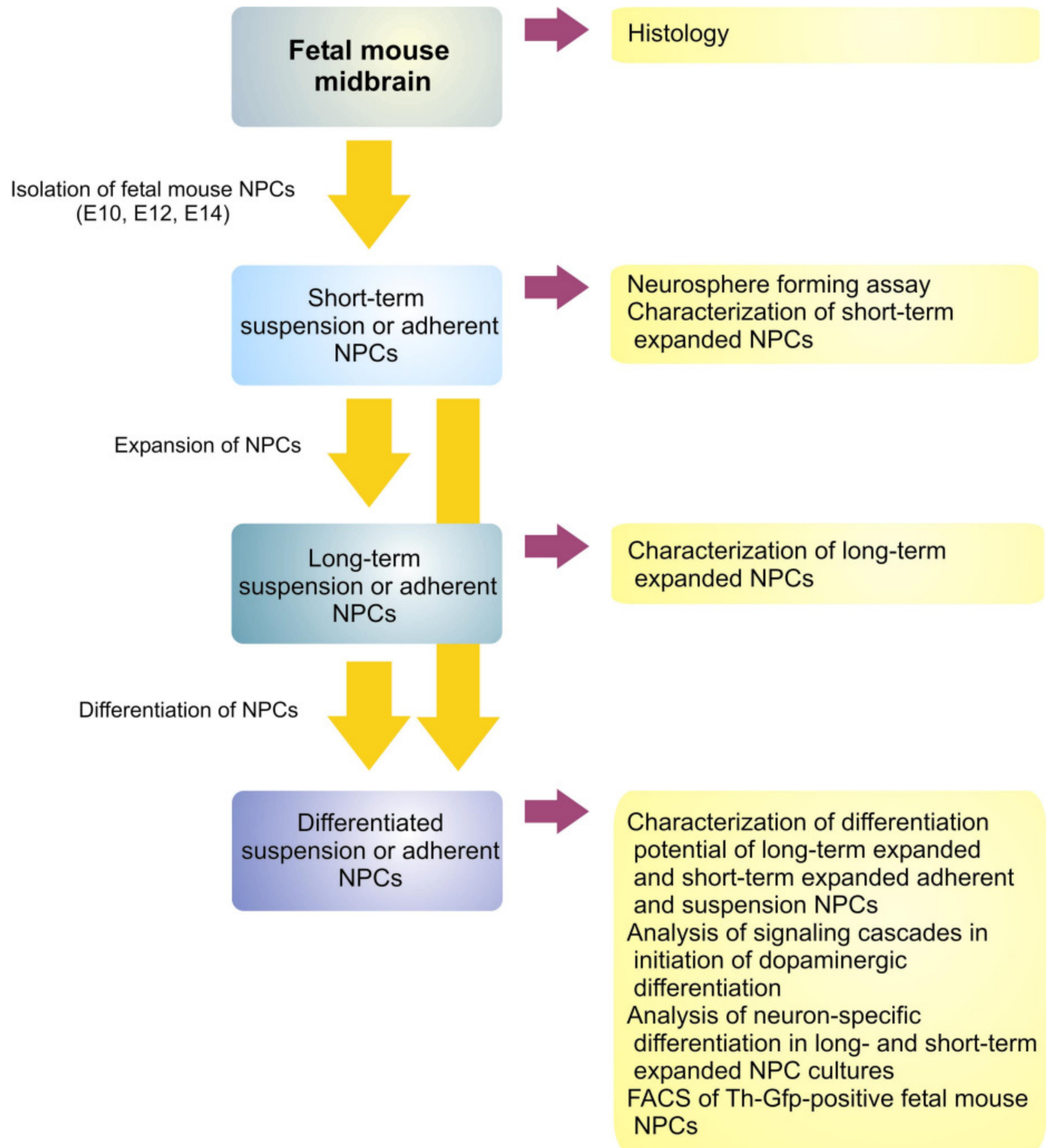
Medium component	Company
32 % F-12 (Nutrient Mixture-Ham-)	Gibco BRL (Tulsa, UK)
65 % DMEM high Glucose	Gibco BRL (Tulsa, UK)
2 % B27	Gibco BRL (Tulsa, UK)
1 % Penicillin/Streptomycin	Gibco BRL (Tulsa, UK)
20 ng/µl Egf	Sigma (St. Louis, USA)
20 ng/µl Fgf-2	Sigma (St. Louis, USA)

2.8. Mice

Male and female wild-type C57Bl6 (25-35 g) were purchased from Charles River, Sulzfeld, and TH-GFP/21-31 (Tg+) mice (114) were a gift from Dr. Kazuto Kobayashi. They were housed, bred and treated according to the guidelines of the European Community (86/609/EEC) and the Society for Neuroscience (January 1985). All experiments were approved by the local ethical committee. Timed pregnant females were obtained by overnight mating. The day of detection of the vaginal plug was considered as E1.

3. Methods

3.1 Flowchart of Experiments



3.2. Cell biology

3.2.1. Isolation of mesencephalic neural progenitor cells

Fetal mesencephalic NPCs were isolated from brain tissue of E14, E12, and E10 mice embryos. Pregnant females were sacrificed according to NIH guidelines and the approval of the local animal care committee. The embryos were isolated and the embryonic midbrain was dissected. After removal of the meninges the tissue samples were cut into small pieces with a scalpel, incubated in 0.1% trypsin for 5 min, triturated and centrifuged for 3 min at 500 x g. The supernatant was removed, 40 mg/ml DNase was added and the sample was homogenized to a quasi single cell suspension by gentle triturating using a fire polished Pasteur pipette. After centrifugation (3 min, 500 x g) the supernatant was discarded and cells were re-suspended in pre-warmed expansion medium EM I containing 20 ng/µl Egf and Fgf-2. 8 ml of the culture were transferred to a 25 cm^2 flask (about 2×10^6 viable cells per flask).

All steps were performed at room temperature.

3.2.2. Cultivation of suspension and adherent neural progenitor cells

Cultures were incubated at 37°C, 5% CO_2, 92% N_2 and 3% O_2 using an incubator equipped with an O_2-sensitive electrode system. The medium was partly changed once or twice a week. For this, cells were transferred into a falcon tube and centrifuged (3 min, 500 x g) to remove the supernatant. After that, they were washed once by resuspension in about 5 ml PBS followed by another centrifugation step (3 min, 500 x g) before 4 ml old medium and 4 ml pre-warmed medium were added and the cells were transferred to a new culture flask again. Growth factors were added every other day. Fetal neurosphere formation was observed after 2-3 days. Neurospheres were

propagated up to 52 weeks, which corresponds to 52 passages. A new passage was generated simultaneously to the procedure for changing the medium, but the culture was distributed to several culture flasks thereby maintaining the initial amount of 2×10^6 viable cells per flask.

Adherent NPCs were seeded at 1×10^6 viable cells per flask on previously Poly-L-Ornithine /Fibronectin coated dishes. In short, coating was done by incubating cell culture flasks with Poly-L-Ornithine solution (0.01%) overnight at 37℃ with agitation. After rinsing three times with PBS Fibronectin solution (4µg/ml) was applied. After another incubation step for at least 3 h at 37℃ and subsequent rinsing with PBS dishes were ready to use or stored at -20℃. Growth factors were added every other day. Cells were passaged once a week. Medium was removed and trypsin was added onto the cells. After a two minute incubation step at 37℃ cells were rinsed off the dish and collected in a centrifuge tube. Cells were spun down and the supernatant was removed. Pre-warmed expansion medium EM I was added and subsequently the cell solution was seeded in new culture flasks to achieve a density of 1×10^6 viable cells per flask.

3.2.3. Neurosphere forming assay

To assess the ability of neural precursor cells to form neurospheres, a neurosphere forming assay was used. Freshly isolated neural precursor cells were counted using a Neubauer counting chamber. A dilution series was prepared from isolated neural precursor cells to obtain mathematically 0.5, 1, 2, 4, 8, 15, 30, 61, 125, 250, 500, 1000 cells per well. The cell dilutions were seeded into a 96-well plate as octuplicates with Egf and Fgf-2 and incubated at 37℃, 5% CO_2, 92% N_2 and 3% O_2 for one week. Growth factors were added every other day.

After one week, the number of negative wells was identified for each dilution. The fraction of negative wells for five individual experiments was quantified. A logarithmic trend line was used to describe the fraction of negative wells against the number of cells initially seeded per well. According to the formula

described by Levkovits and Waldmann (94) the number of cells needed to form one neurosphere was determined at 0.37 = fraction of negative wells (y).

3.2.4. In vitro *differentiation of neural precursor cells*

NPCs were differentiated by plating them onto poly-D-lysine-coated (50µg/ml) 24 well-plates or 6 well-plates at 50,000-150,000 cells per cm^2 in standard medium without mitogens. In short, poly-D-lysine (aqueous solution) was pipetted into culture dishes and incubated for 1 h at 37°C or overnight at room temperature. After rinsing once with HBSS and four times with PBS the plates were immediately used for differentiation experiments. Neurosphere cultures in EM I medium were prepared and 500 to 2000 µl were filled into culture dishes according to their size. The following substances were used to induce differentiation: Interleukin (IL)-1 (100 pg/ml), Fgf-8 (100 ng/ml), Shh (500 ng/ml) alone or in combination (Fgf-8/Shh) and in combination with Forskolin (50 µM). The cells were allowed to differentiate for up to 14 days at 37˚C in a humidified atmosphere at lowered O_2 conditions of 5% CO_2, 92% N_2 and 3±2% O_2. Half of the medium was changed and cytokines/growth factors were supplemented every 3-4 days.

3.2.5. Flow Cytometry

Isolated fetal NPCs (E14) from pregnant Th-Gfp mice in a single cell suspension were filtered and subsequently processed by a FACSCalibur® FACS Sorter and analyses were performed with the Cellquest software (both from Becton Dickinson, Franklin Lakes NJ) in collaboration with the Max-Planck-Institute for Cell Biology and Genetics. A Th-Gfp negative fraction and a mixed fraction were obtained and cultivated separately for one to two weeks and then subjected to differentiation.

3.2.6. Immunocytochemistry

Immunocytochemistry allows localizing proteins within a cell by exploiting the principle of antibodies which bind specifically to the protein of interest. The primary antibody is then recognized by a secondary antibody linked to a fluorophore, thereby allowing specific visualization of the target protein.

Cell culture plates were equiped with glass cover-slips before coating. Cells were cultivated on coated cover-slips according to the experimental design. At the end of the experiment the cells were washed once with PBS and subsequently fixed for 30 seconds using “Accustain” solution. After rinsing three times with PBS, parts of the surface not covered with cells were blocked against unspecific antibody labeling using blocking solution (3% donkey-serum in PBS containing 0.2% Triton) for 1.5 h. After that, primary antibodies were added and incubated overnight at 4℃. Subsequently, cells were rinsed three times with PBS and incubated with the corresponding fluorophore-labeled secondary antibodies (diluted 1:500 in PBS) for 1h at a dark place. After the incubation with the secondary antibodies, cells were rinsed three times with PBS and cell nuclei were counterstained with Hoechst 33342 staining solution (0.75 µl/ml) for 3 min. After rinsing again for three times with PBS, cover-slips were removed from the culture dish and transferred onto microscopy slides using mounting medium. Images were captured using a fluorescence microscope.

Unless indicated differently, all incubation steps were performed at room temperature.

3.3. Histology

3.3.1. Perfusion

Pregnant females at E14 post-fertilization were perfused to obtain E14 embryonic brain sections. Fix and wash bottle lines were cleared of any air bubbles. Wash solution was run through the canula to remove any fix

from lines and was then set at a slow drip. The anesthetized animal was placed on its back on a styropor block over a sink. Limbs were spread and each paw secured to the block.

A cut along the sternum ~8 cm long was made; scissors were used to cut the diaphragm laterally on both sides and toward the head across ribs and parallel to lungs. The descending aorta was clamped. With a mosquito hemostat, one lung was reflected to locate the descending aorta as it runs along the spinal column. The aorta was clamped. A small pair of rat tooth forceps was used to grasp the ventral tip of the heart. The left ventricle was pierced with a scalpel, allowing the blade to penetrate far enough to make the slit large enough for the canula tip. The blade was quickly removed to prevent spurts from the long incision. The canula was inserted and directed up through the left ventricle into the ascending aorta. The aorta was clamped, holding the tip of the canula in place. After a brief wash step the fix line clamp and perfusion tube clamp were fully opened. The right auricle was punctured, allowing the escape of return circulation.

After perfusion, embryos were dissected, post-fixed overnight, incubated in growing concentrations of sucrose solution and finally quick-frozen and stored at -80℃.

3.3.2. Immunohistochemistry

Immunohistochemistry allows localizing proteins within a tissue by exploiting the principle of antibodies which bind specifically to the protein of interest. Such a primary antibody is then recognized by a secondary antibody linked to a fluorophore, thereby allowing specific visualization of the target protein.

Whole embryos or embryonic brains were embedded in Tissue Tek O.C.T. Compound, and 20-30 µm thick sections were applied onto microscope slides, dried, and stored at 4℃ until further use.

The specimens were outlined on the slides with a Pap Pen to prevent the spilling of liquids. The blocking procedure was done with 3% donkey-

serum in PBS containing 0.2% Triton for 1.5 h. After that, primary antibodies were added over night at 4°C and the specimens were stored in a moist chamber. Subsequently, the sections were rinsed three times with PBS and further incubated with the corresponding fluorophore-labeled secondary antibodies (diluted 1:500 in PBS) for 60-90 minutes in a dark humid chamber. Specimens were rinsed with PBS three times and cell nuclei were counterstained with Hoechst 33342 staining solution (0.75 µl/ml PBS) for 3 min. After carefully rinsing with PBS, specimens on microscopy slides were covered with mounting medium and cover slips, which were sealed to the slides with nail polish. Images were captured using fluorescence microscopes.

3.4. Molecular biology

3.4.1. RNA Isolation

Isolation of RNA was performed with the "Rneasy Mini Kit" (Qiagen) followed by treatment with RNase-free DNase according to the manufacturer's manual. In this procedure, RNA is bound to a silica gel membrane and washed several times to remove contaminants and enzyme inhibitors before elution.

For determining RNA concentration, absorbance was measured at 260 nm. At this wavelength, absorption of 1 corresponds to a concentration of 40 µg/ml. To find out the extent of potential contaminations, absorbance was also measured at 230 and 280 nm. For a pure sample, the ratio of absorption at 260 and 280 nm should be greater than 2.0, whereas the ratio of absorbance at 260 and 230 nm should be greater than 1.7.

3.4.2. DNA isolation

To isolate genomic DNA (gDNA) from Th-Gfp mice, the end of the tail was cut off and stored if necessary at -80°C. DNA was isolated with DNeasy Blood and Tissue Kit according to the manufacturer's manual. In short, the

tissue was lysed using Proteinase K and after appropriate buffering the DNA was adsorbed at a silica-based membrane in the presence of high concentrations of chaotropic salt, which remove water from hydrated molecules in solution.

During centrifugation, DNA was selectively bound to the membrane as contaminants passed through. Remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was finally eluted in water and stored at -80℃.

3.4.3. Genotyping (TH)

Genotyping PCR was done to assure the correct genotype of Th-Gfp animals. A positive control, a negative control and sample DNAs were included into the analysis.

For one sample, the following pipetting scheme was used:

Table 1: Pippeting scheme used for Genotyping PCR.

10x Taq Buffer	2.5 µl
5x Taq M Enhancer	2.5 µl
2mM dNTP's	2.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq Polymerase	0.7 µl
gDNA	4 µl
Ultra-Pure Water	10.8 µl
Total	25 µl

The following PCR programme was used to amplify the gDNA fragments:

Table 2: Cycling protocol used for Genotyping PCR.

Step1	2’	95℃
Step2	30”	94℃
Step3	30”	56℃
Step4	45”	72℃
Step 2 to 4 were repeated 35 times.		
Step5	10’	72℃.

Agarose gel electrophoresis confirmed a fragment of 450 bp for Th-Gfp-positive animals. Samples from Th-Gfp-negative animals did not display a fragment.

3.4.4. Quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) is the most sensitive and reliable procedure for gene expression analyses. Its wide dynamic range makes qRT-PCR the method of choice for the simultaneous quantification of both rare and abundant genes in the same sample.

QRT-PCR was performed with the “Brilliant® SYBR® Green QRT-PCR Master Mix Kit” (Stratagene) according to the manufacturer’s manual. The method is based on the principle that fluorescent dye SYBR Green fluoresces when bound to double-stranded DNA. In the following, the fluorescence response can be monitored in a linear fashion as PCR product is generated over a range of PCR cycles. Relative concentrations of DNA present during the exponential phase of the reaction are then determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background is defined, whereas the cycle at which the fluorescence from a sample crosses the

threshold is called the threshold cycle, C_t. This value was used for the analysis of gene expression.

1 µl (approximately 200 ng) of total RNA was reverse transcribed and subsequently amplified using 0.5 µM of both sense and antisense primer. After amplification, melting curves of the qRT-PCR products were acquired to demonstrate product specificity. The following cycling protocol was used:

Table 3: Cycling protocol used for quantitative qRT-PCR

Cycles	Duration	Temperature	Procedure
1	25 min	50°C	first-strand cDNA synthesis
1	15 min	95°C	initial denaturation of cDNA /activation of DNA polymerase
35	15 sec	95°C	denaturation of DNA
	20 sec	60°C	binding of primers to DNA
	30 sec	72°C	elongation of primers to synthesize complementary strand
1	1 min	95°C	denaturation of PCR products
1	30 sec	55°C	annealing of PCR products to generate dissociation curves

The results were expressed relative to the housekeeping gene *Hmbs* (hydroxymethylbilane synthase), whose expression did not vary under the experimental conditions used. Quantification was performed using the formula of the comparative threshold cycle ($\Delta\Delta C_t$ method) 2^ (C_t [housekeeping gene] - C_t [target gene]) described by Livak et al.(102).

3.5. Other methods

3.5.1. Cell counting and statistics

For quantification of the percentage of cells producing a given marker a total of 100 to 1000 cells were counted per marker and the number was determined relative to the total number of DAPI-labeled nuclei. Statistical comparisons were made by Dunnett's *t*-test and ANOVA. If data were not normally distributed, a non-parametric test (Mann-Whitney U-test) was used for comparisons of results. All data were presented as mean ± SEM.

4. Results

4.1. Characterization of neural progenitor cells

Tissue-specific stem cells are a suitable model for studying stem cell behavior and exploring the potential of stem cells for their application in regenerative therapies. They have never been reported to retain tumorigenic potential and are able to generate neurons and glia after cultivation *in vitro*. To investigate the regenerative potential of mouse fetal neural progenitor cells (NPCs) their characteristics were studied in detail and the signaling cascades involved in their *in vitro* differentiation were explored.

Dopaminergic neurons arise from embryonic day (E) 10.5 to E12.5 in the mouse embryo. Fetal NPCs were isolated from time-mated C57Bl/6 mice at E10, E12 and E14 to explore differences in the dopaminergic potential of *in vitro* NPCs. They were subsequently grown as a typical neurosphere culture and as a monolayer on Poly-L-Ornithin/ Fibronectin coated dishes for at least 8-12 weeks prior to experiments.

E14 fetal mouse NPCs were characterized by assessment of their morphology, a neurosphere forming assay and existence of diverse neural and stem cell markers on mRNA as well as protein levels (**Fig.3**). The morphology of cells in neurosphere cultures was round and a typical neurosphere contained 20 to 200 cells, while cells in adherent culture appeared mostly spindle-like as other adherent cell cultures (**Fig. 3A**). Cells in the middle of spheres were frequently differentiated or apoptotic, probably due to limited access to growth factors as reported by Svendsen and Smith (172). In adherent cultures only very few cells displayed protrusions or branched extensions showing a differentiated phenotype. It was important to re-plate monolayer NPC cultures as soon as sub-confluent status was reached, because the morphology of some cells appeared to change in cultures with a high density towards differentiated phenotypes.

A neurosphere forming assay was performed for E14 suspension culture NPCs demonstrating that in a typical experiment 75 cells were necessary to form one neurosphere (**Fig. 3B**). The outcome of this

experiment varied strongly, because dilution series were very sensitive to personal errors and the survival of the cells was indirectly proportional to the length of remaining at room temperature. Cultures were usually terminated at 6 to 12 months, but at that time no drastic slowing of the cell-cycle or obvious phenotypic changes were observed by means of re-plating frequency of the culture. It has previously been shown that fetal cells could be cultured for up to 120 days in serum-free medium with a logarithmic increase in cell number and no evidence of gross chromosomal abnormality (105).

QRT-PCR data demonstrated high expression of the stem cell marker Nestin (92) and epidermal growth factor receptor (*Egf-R*) in adherent culture compared to suspension culture (3.4±1.7 compared to 0.4±0.2 (p≤0.01, n=6) and 2.5±0.98 compared to 0.6±0.44 (p≤0.05, n=4) relative to the housekeeping gene *HMBS*) of E14 midbrain NPCs (**Fig. 3C**). The neural marker *Tuj1* was expressed at low levels compared to the housekeeping gene (<1), however more prominent in suspension than in adherent NPCs (0.46±0.23 compared to 0.22±0.07; p≤0.05, n=4). *Th* (*tyrosine hydroxylase*) mRNA as a marker for dopaminergic neurons was not present in either culture. The glial markers *Gfap* (glial fibrillary acidic protein) and *MBP* (myelobasic protein) were expressed at low levels compared to the housekeeping gene (<1) in both NPC cultures. Suspension culture cells contained significantly more mRNA for the stem cell markers *Sox-2* (4.1±1.3 compared to 1.8±0.4; p≤0.01, n=4), *Musashi* (*Msi*; 1.3±0.35 compared to 0.4±0.05; p≤0.05, n=4) and *Prominin* (*Prom*; 0.62±0.47 compared to 0.08±0.03, p≤0.05, n=4), whereas *Nkx2.2* (a marker for neuroepithelial neuroblasts) and *Ngn-2* (marker for ventricular zone (VZ)/ subventricular zone (SVZ) progenitors) were transcribed at higher levels in adherent NPCs (4.2±1.5 compared to 1.0±0.3 (p≤0.05, n=4) and 0.14±0.02 compared to 0.09±0.03 (p≤0.01, n=4), respectively). Equally transcribed in both cultures were *Otx2* (VZ/SVZ progenitors) and *En1* (ventral midbrain dopaminergic precursors) at levels equitable to the housekeeping gene. *Otx1* (neuroepithelial neuroblasts), *Aldh1a1* (VZ/SVZ progenitors) and *En2* (ventral midbrain dopaminergic precursors) were equally transcribed, but at low levels (<1) compared to the housekeeping gene. *FoxA1* and *Nr4a2*

(ventral midbrain dopaminergic precursors), as well as *FoxA2* and *Lmx1a* (VZ/SVZ precursors), and *Msx1*, *Lmx1b* and *Pitx3* (ventral midbrain dopaminergic precursors) were not expressed at all (**Fig. 3C**). QRT-PCR is a very sensitive method that produced variable results, especially for mRNA that was very low or high abundant compared to the housekeeping gene of use. Alternative housekeeping genes (*β-Actin*, *Gapdh*) were used, but they

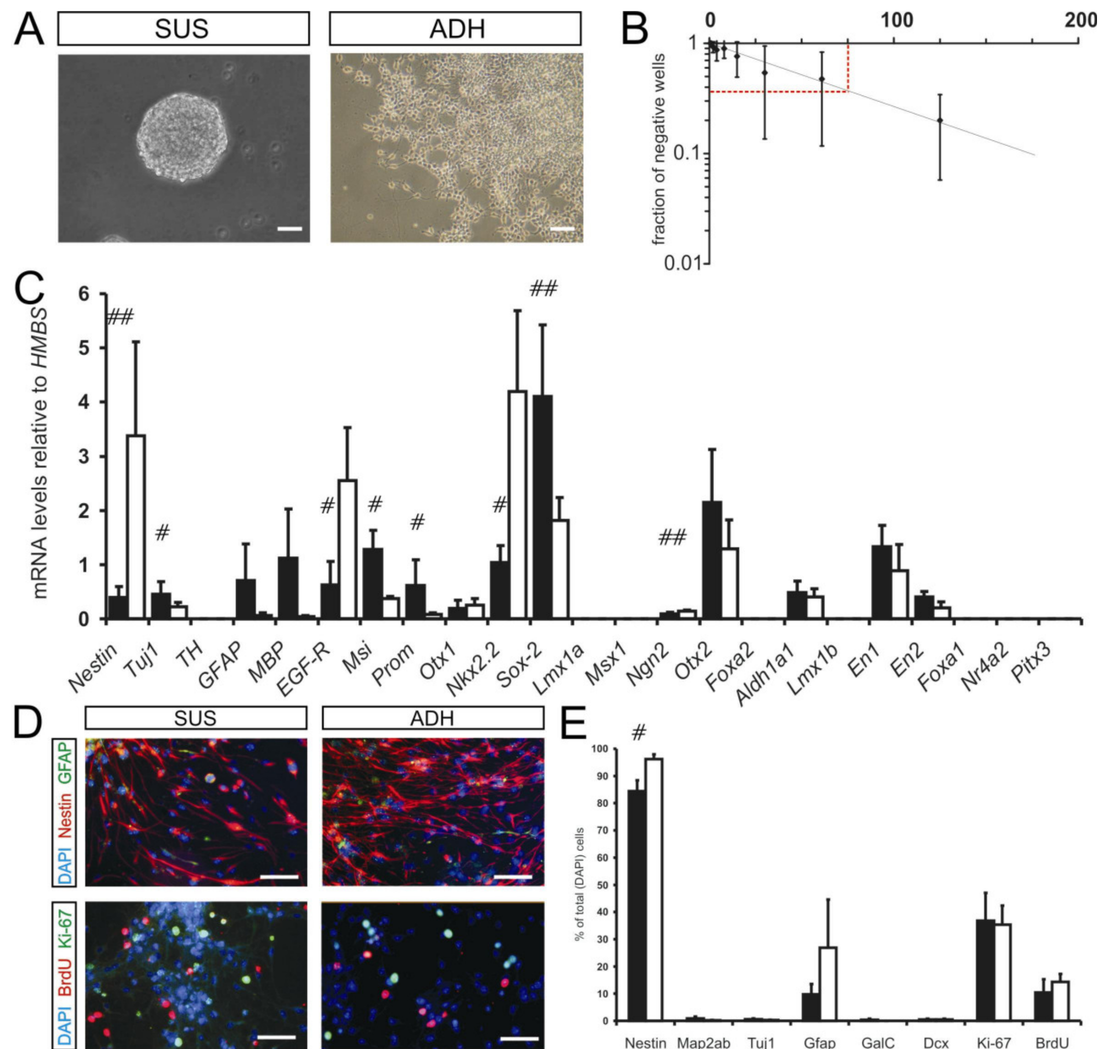


Fig. 3 Characteristics of E14 fetal mouse NPCs. **A**: Brightfield images of suspension and adherent NPCs. Scale bar: 50 µm. **B**: Neurosphere forming assay. The fraction of negative wells (no neurosphere formation) is shown versus initially plated cell number. Linear regression allows theoretical calculation of cells needed to form a neurosphere (at 0.37 of fraction of negatve wells). **C**: qRT-PCR of suspension (black) and adherent (white) NPC culture. **D**: Immunocytochemistry of E14 suspension (left panels) and adherent (right panels) NPCs. Scale bar: 50µm. **E**: Quantitative analysis of immunocytochemistry data of E14 suspension (black) and adherent (white) NPCs. Data are expressed as mean ± s.e.m. # - $p \leq 0.05$, ## - $p \leq 0.01$.

varied strongly in samples of different brain areas and NPC types and were thus excluded from analysis.

Adherent E14 NPC cultures were almost completely (96±1.7%) positive for the neural progenitor marker Nestin, whereas only 84±4% of the suspension culture total cells expressed Nestin (**Fig. 3D, E**) confirming qRT-PCR data.

In both cultures about 36±10% of the cells stained for the proliferative marker Ki-67, and after incubation with BrdU for 20 h the same amount of cells were positive for BrdU, suggesting that the proliferating part of the culture (Ki-67-positive) doubled in 20 h (BrdU-positive) in adherent as well as suspension NPCs (**Fig. 3D, E**). Interestingly, not all cells that were Ki-67-positive were also BrdU-positive and not all BrdU-positive cells were Ki-67-positive. This suggested that asymmetric cell divisions producing one progenitor and one cell becoming post-mitotic were going on in the cultures. However, this observation was at least partly veiled by proliferating daughter cells that at the time of fixation were residing in the early G1-phase of the cell cycle, staining for BrdU but not for Ki-67, suggesting symmetric divisions.

The amount of Gfap-positive cells varied strongly under both culture conditions between 10 and 40% of the total amount of cells (**Fig. 3D, E**). Astroglia appeared to be sentinel cells, the number of astroglia always correlated with a healthy look of an NPC culture (personal observation). In both cultures only few cells were positive for the postmitotic markers Dcx (0.5±0.3), Tuj1 (0.4±0.2), Map2ab (suspension NPCs: 0.9±0.6, adherent NPCs: 0.2±0.1) and GalC (suspension NPCs: 0.4±0.4, adherent NPCs: 0±0) (**Fig. 3E**). No cells stained positive for tyrosine hydroxylase (Th; data not shown).

The characterization of long-term expanded E14 fetal mouse NPCs demonstrated that they were a rapidly dividing cell culture with a constant phenotype. They expressed the neural precursor marker Nestin and transcribed numerous markers of stem cells and neuronal development that marked them as a mixed culture of neuroepithelial, VZ/SVZ, and ventral midbrain committed progenitors.

E12 mouse suspension and adherent NPCs transcribed equally low amounts (<1 relative to housekeeping gene *HMBS*) of *Nestin* and *Prominin* (*Prom*) mRNA (**Fig. 4A**). *Gfap* and *Egf-R* mRNA were abundant in adherent NPCs but only present at low levels in suspension cells (0.5±0.44 compared to 0.05±0.01 ($p \leq 0.05$, n=6) and 2.5±0.38 compared to 0.09±0.03 ($p \leq 0.01$, n=5), respectively). Beta-III-Tubulin (*Tuj1*) mRNA was transcribed at a higher level in suspension NPCs (1.4±0.38 compared to 0.6±0.3; $p \leq 0.05$, n=5). *Th, Nr4a2 and Pitx3* mRNA were not expressed in suspension or adherent E12 NPCs. Suspension and adherent E12 NPCs both expressed Nestin on the protein level (**Fig. 4B,C**), but adherent cells were almost completely (97±0.7%) positive, whereas only 83±5% of suspension cells showed Nestin immunoreactivity (**Fig. 4B, C**). E12 long-term propagated NPCs were negative for Th (**Fig. 4C**). In summary, fetal (E12) NPCs were a rapidly dividing population that displayed Nestin expression. Markers for

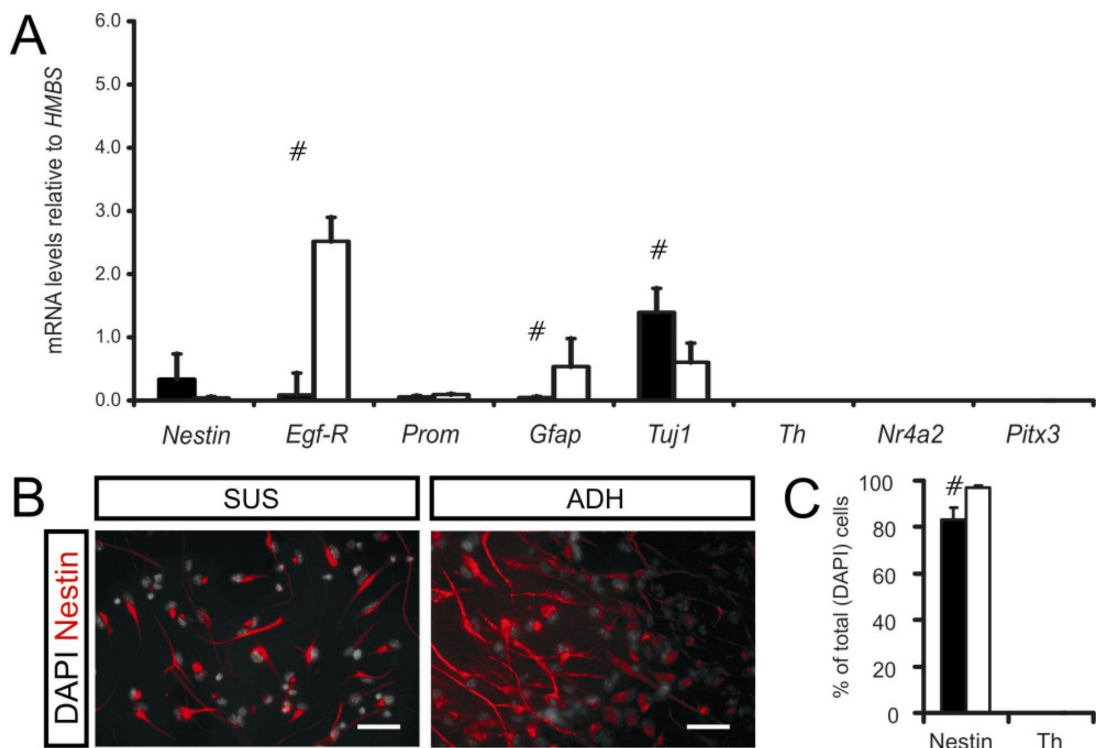


Fig. 4 Characteristics of E12 fetal mouse NPCs. **A**: Relative mRNA levels of suspension (black) and adherent E12 fetal mouse NPCs. **B**: Nestin immunostain (red) of suspension (left panel) and adherent (right panel) NPCs. Nuclei were counterstained with DAPI (white). Scale bar: 50 µm. **C**: Quantitative analysis of immunocytochemical analysis of Nestin and Th in suspension (black) and adherent (white) E12 fetal mouse NPCs. Data are expressed as mean ± s.e.m. # - $p \leq 0.05$, ## - $p \leq 0.01$.

dopaminergic differentiation (*Th, Nr4a2* and *Pitx3*) were absent on the mRNA level.

E10 suspension and adherent NPCs expressed *Nestin* mRNA but on a low level (<1 compared to the housekeeping gene *HMBS*). Interestingly, E10 NPCs transcribed fairly high amounts (>1 compared to housekeeping gene) of *Prominin* (*Prom*) mRNA. Adherent E10 NPCs expressed more *Gfap* and *Egf-R* mRNA than the suspension culture (1.5±0.9 compared to 0.2±0.1 (p≤0.05, n=4) and 2.26±0.76 compared to 0.02±0.004 (p≤0.05, n=4) respectively) (**Fig. 5A**). They also displayed low levels (<1 compared to housekeeping gene) of *Tuj1* mRNA. *Th, Nr4a2* and *Pitx3* mRNA were not transcribed in E10 mouse neural progenitors. E10 adherent NPCs were almost homogeneously (97±1%) positive for Nestin, whereas the suspension

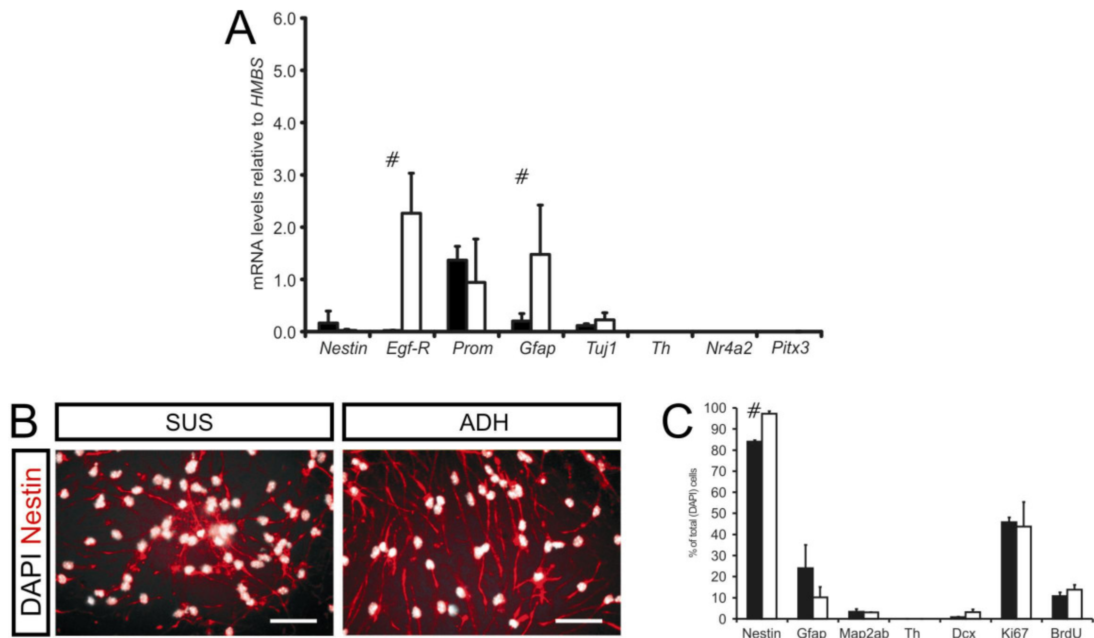


Fig. 5 Characteristics of E10 fetal mouse NPCs. **A**: Relative mRNA levels of suspension (black) and adherent E10 fetal mouse NPCs. **B**: Nestin immunostain (red) of suspension (left panel) and adherent (right panel) NPCs. Nuclei were counterstained with DAPI (white). Scale bar: 50 µm. **C**: Quantitative analysis of immunocytochemical results of suspension (black) and adherent (white) E10 fetal mouse NPCs. Data are expressed as mean ± s.e.m. # - p≤0.05, ## - p≤ 0.01.

culture harbored only 84±0.7% Nestin-positive cells (**Fig. 5B, C**). Again, adherent and suspension NPC cultures displayed the same amount (45±10%) of proliferating cells as shown by Ki-67 staining (**Fig. 5C**). BrdU was incorporated by 11±2% (suspension) and 14±2% (adherent) of the

cultures when added for 6h, suggesting a slightly higher proliferative potential than E14 NPCs. Gfap was expressed in varying amounts of cells in both NPC types. Map2ab (3±1%) and Dcx (2±1%) were rarely detected in both culture systems. Th immunoreactivity was not present (**Fig. 5C**). E10 long-term expanded NPCs were a rapidly dividing cell population that was largely Nestin-positive and infrequently showed markers of differentiated neural cell types. Markers of dopaminergic differentiation were absent.

The comparison of NPCs that were generated from three developmental stages (E10, E12, and E14) and cultivated in two different ways on the mRNA level demonstrated that *Nestin* mRNA production was highest in E14 adherent NPCs and much lower at the other stages irrespective of their cultivation method (**Fig. 6**). *Egf-R* mRNA was higher in adherent cells compared to suspension cells from all embryonic stages. *Prominin* (*Prom*) was significantly expressed only in E10 NPCs and - to a lesser extend - in

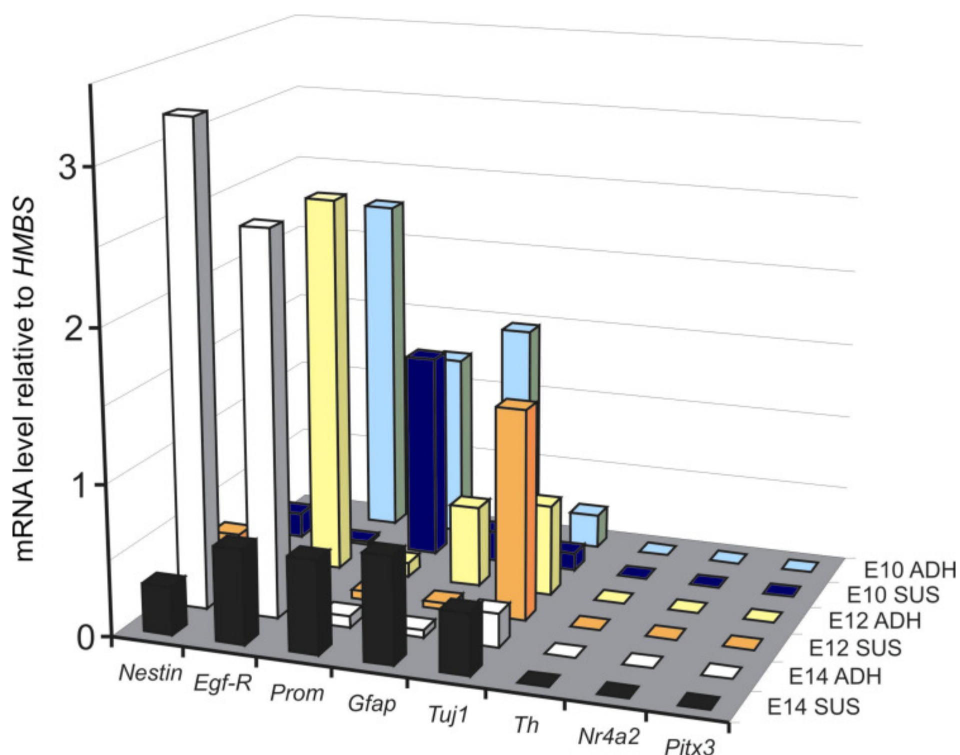


Fig. 6 Comparison of relative mRNA levels of NPCs from three different developmental stages (E14 (front), E12 (middle) and E10 (back)) and suspension (black, orange or blue bars) or adherent (white, yellow or light blue bars) culture conditions.

E14 suspension NPCs compared to other NPCs, suggesting that NPCs do retain at least some of their temporal identity *in vitro*. Amounts of *Gfap* mRNA varied strongly and were transcribed at equal levels in respect to the housekeeping gene only in E14 suspension and E12 adherent NPCs and were low abundant (<1 compared to housekeeping gene *HMBS*) under all other conditions. *Tuj1* mRNA levels varied from low (<1) to normal (~1 compared to *HMBS*) at E14 to E12, but were hardly detectable at embryonic day (E) 10. *Th (tyrosine hydroxylase)*, *Nr4a2,* and *Pitx3* mRNA were not transcribed in long-term expanded fetal mouse NPCs from any embryonic stages.

In summary, long-term cultivated NPCs from embryonic stages E10 to E14 are a self-renewing, rapidly dividing source of mixed progenitors that express Nestin and infrequently show markers for differentiated neural cells like *Tuj1* (neuronal), *Gfap* (astroglial) and *MBP* (oligodendroglial) but not markers of dopaminergic differentiation as *Nr4a2*, *Pitx3* or *Th*.

4.2. Early differentiation of fetal mouse mesencephalic NPCs.

Long-term cultivated NPCs displayed characteristics of a mixed stem/progenitor culture and had the potential to self-renew for a long period of time *in vitro*. To investigate whether they also retained the ability to differentiate into neurons and glia after prolonged periods in culture, the response to known factors important for dopaminergic differentiation was tested. E14 neural progenitor cells were expanded as a neurosphere culture in serum-free medium with mitogens Fgf-2 and Egf for at least 8 to 12 weeks prior to experiments. For differentiation experiments NPCs were plated on Poly-D-Lysine (PDL) coated cell culture dishes. Interleukin-1 (IL-1), Forskolin (a cAMP stabilizer), IL-1 and Forskolin, the combinations Fibroblast growth factor-8 (Fgf-8) and Sonic hedgehog (Shh) and Fgf-8/Shh/Forskolin or no factors were added to serum-free medium. Early differentiation was assessed by qRT-PCR of key regulators of dopaminergic differentiation and by cell-based ELISA of critical signaling molecules

involved in dopaminergic differentiation of *in vitro* NPCs. At 3h after differentiation initiation a downregulation of *Th* mRNA was observed in IL-1, Forskolin and IL-1/Forskolin treated cultures (0.34±0.07 ($p \leq 0.01$, n=3), 0.52±0.2 ($p \leq 0.05$, n=3) and 0.33±0.19 ($p \leq 0.01$, n=3), respectively) compared to control (no factors added), but not in Fgf-8/Shh and Fgf-8/Shh/Forskolin treated cultures (**Fig. 7A**). The orphan receptor *Nurr1* (*Nr4a2*) was significantly upregulated after 3h only in IL-1/Forskolin treated cultures

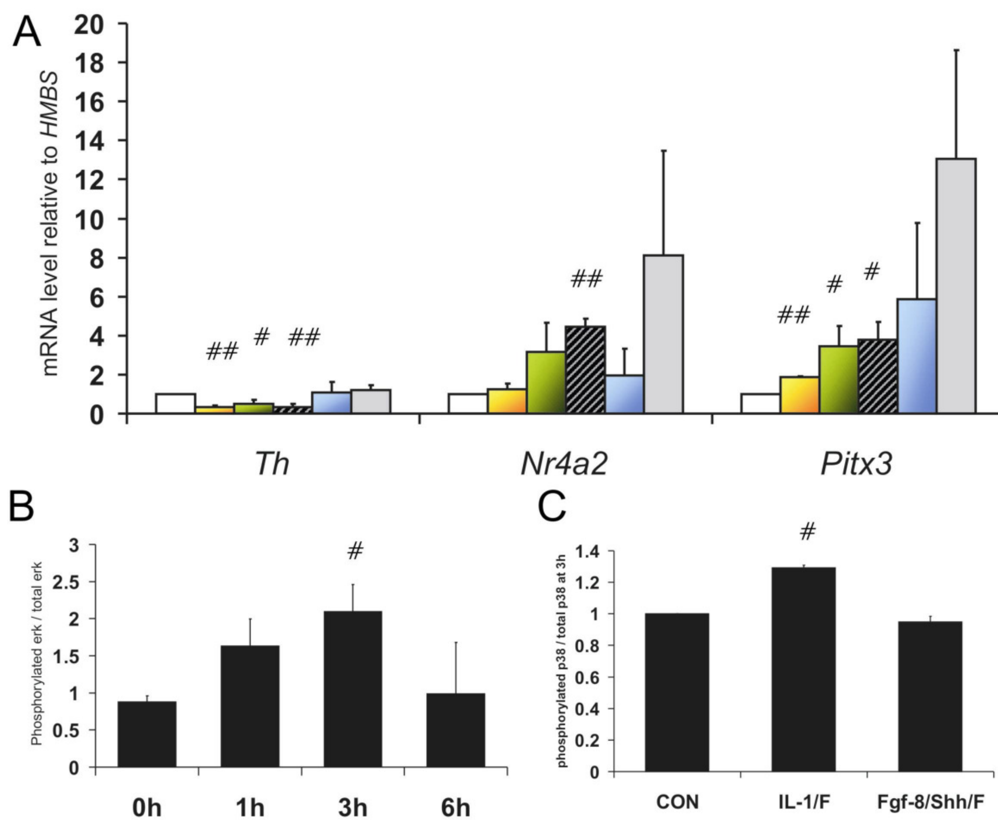


Fig. 7 Early differentiation characteristics of E14 fetal mouse NPCs. **A**: Relative mRNA levels of differentiated samples (no factor: white, IL-1 only: yellow, Forskolin only: green, IL-1 and Forskolin: diagonal stripes, Fgf-8 and Shh: blue, Fgf-8, Shh and Forskolin: grey). **B**: Cell-based ELISA of activated signaling cascade molecule Erk1/2 in IL-1 treated differentiated samples. **C**: Cell-based ELISA of activated signaling cascade molecule p38 at 3h of Control (CON = no factor), IL-1 and Forskolin (IL-1/F) and Fgf-8, Shh and Forskolin (Fgf-8/Shh/F) treated differentiated E14 fetal mouse NPCs. Data are expressed as mean ± s.e.m. # - $p \leq 0.05$, ## - $p \leq 0.01$.

(4.44±0.41 ($p \leq 0.05$, n=3) compared to control (=1). *Pitx3* mRNA was upregulated after 3h in IL-1, Forskolin and IL-1/Forskolin treated samples compared to control (1.87±0.02 ($p \leq 0.01$, n=3), 3.43±1.07 ($p \leq 0.05$, n=3) and

3.77±0.94 ($p \leq 0.05$, n=3), respectively). This early response may have been mediated by the activation of Erk1/2 and p38 MAPK signaling cascades. They were significantly upregulated by IL-1/Forskolin after 3h compared to untreated samples (**Fig. 7B, C**). Fgf-8/Shh/Forskolin did not lead to an activation of the p38 signaling cascade (**Fig. 7C**).

In summary, after 3h NPCs respond to IL-1 and/or Forskolin-mediated differentiation by activating Erk1/2 and p38 signaling cascades and transcription of the two key fate-determining factors for dopaminergic differentiation: *Nr4a2* and *Pitx3*.

4.3. Late differentiation of fetal mouse mesencephalic NPCs.

Long-term cultivated NPCs had the potential to self-renew and respond to known factors involved in *in vitro* differentiation of dopaminergic neurons. Another hallmark of stem/progenitor cells is the differentiation into all three neural lineages after *in vitro* cultivation. To investigate the ability of long-term cultivated E14 NPCs to form neurons and glia, the cells were cultivated as neurosphere or monolayer cultures for at least 8-12 weeks prior to experiments, replated or plated on PDL-coated dishes and treated with no factors, IL-1/Forskolin or Fgf-8/Shh/Forskolin for *in vitro* differentiation. Late differentiation characteristics were monitored after 7 days by qRT-PCR and immunocytochemical visualization of markers important for neural differentiation.

After an interval of 7 days, differentiated suspension culture samples did not display *Nestin* mRNA transcription compared to NPCs (0.4±0.2; $p \leq 0.05$, n=3), irrespective of the factors used for differentiation (**Fig. 8A**). *Pitx3* mRNA was significantly upregulated under control conditions (1.09±0.39; $p \leq 0.05$, n=3), IL-1/Forskolin treatment (1.55±0.15; $p < 0.01$, n=3) and Fgf-8/Shh/Forskolin treatment (1.19±0.33; $p \leq 0.05$, n=3) compared to NPCs where Pitx3 was not expressed. *Nr4a2* transcription was not significantly activated in differentiated samples after 7 days compared to NPCs. The neuronal marker *Tuj1* was upregulated on the transcriptional level in control (0.73±0.13; $p \leq 0.05$, n=3) and IL-1/Forskolin samples in

suspension cultures (0.54±0.09; p≤0.05, n=3) compared to NPCs (0.27±0.09).

In adherent NPCs differentiated by mitogen removal (no factor control, 0.07±0.03; p≤0.05, n=3) and IL-1/Forskolin treatment (0.10±0.04; p≤0.05, n=3) *Nestin* transcription was reduced after 7 days compared to NPCs (3.4±1.7) (**Fig. 8B**). Fgf-8/Shh/Forskolin treatment (0.62±0.36; n=2) did not lead to a significant reduction of *Nestin* mRNA levels. On the other

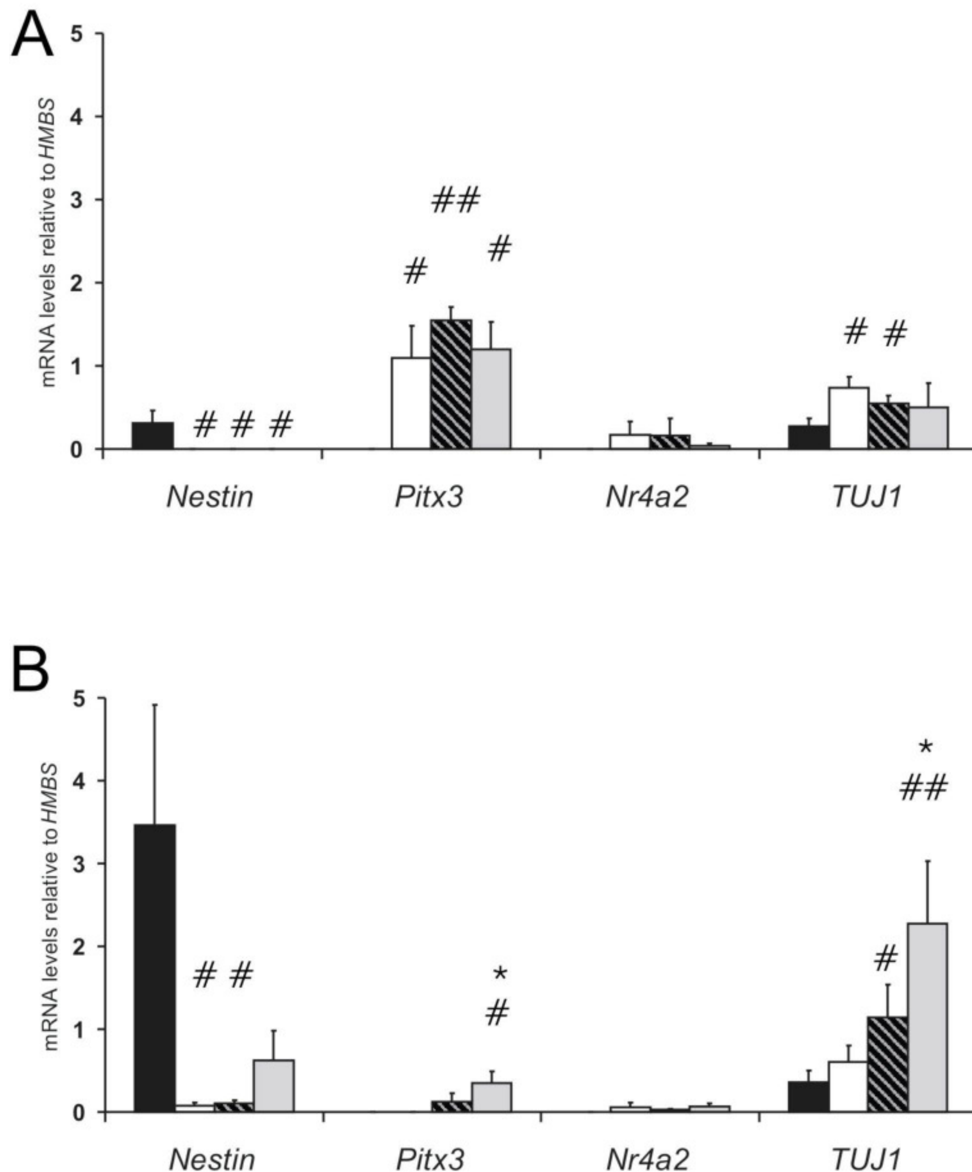


Fig. 8 Characteristics of E14 fetal mouse NPCs after 7d of differentiation. **A**: Relative mRNA levels of differentiated *suspension* samples (no factor (control): white, IL-1 and Forskolin: diagonal stripes, Fgf-8, Shh and Forskolin: grey) relative to NPCs (black). **B**: Relative mRNA levels of differentiated *adherent* samples (no factor (control): white, IL-1 and Forskolin: diagonal stripes, Fgf-8, Shh and Forskolin: grey) relative to NPCs (black). Data are expressed as mean ± s.e.m. # - p≤0.05 (to NPCs), ## - p≤ 0.01 (to NPCs), * - p≤0.05 (to control), ** - p≤ 0.01 (to control).

hand only Fgf-8/Shh/Forskolin treatment (0.34±0.14; p≤0.05, n=3) led to an upregulation of *Pitx3* mRNA after 7 days compared to NPCs as well as control (no factor) differentiated samples where *Pitx3* was not expressed. *Nr4a2* mRNA was not regulated at the 7 days timepoint in adherent cells. *Tuj1* mRNA was upregulated in IL-1/Forskolin treated samples (1.19±0.4; p≤0.05, n=3) compared to NPCs (0.36±0.14) and in Fgf-8/Shh/Forskolin treated samples compared to NPCs (2.27±0.75 compared to 0.36±0.14; p≤0.01, n=3) as well as control differentiation (2.27±0.75 compared to 0.6±0.2; p≤0.05, n=3).

Immunocytochemistry revealed that adherent as well as suspension culture E14 NPCs differentiated into neurons (Tuj1, Map2ab), astroglia (Gfap) and oligodendrocytes (GalC) after removal of mitogens and plating on PDL-coated dishes (**Fig. 9**). E10 and E12 NPCs also differentiated into all three cell-types of the nervous system (data not shown). After

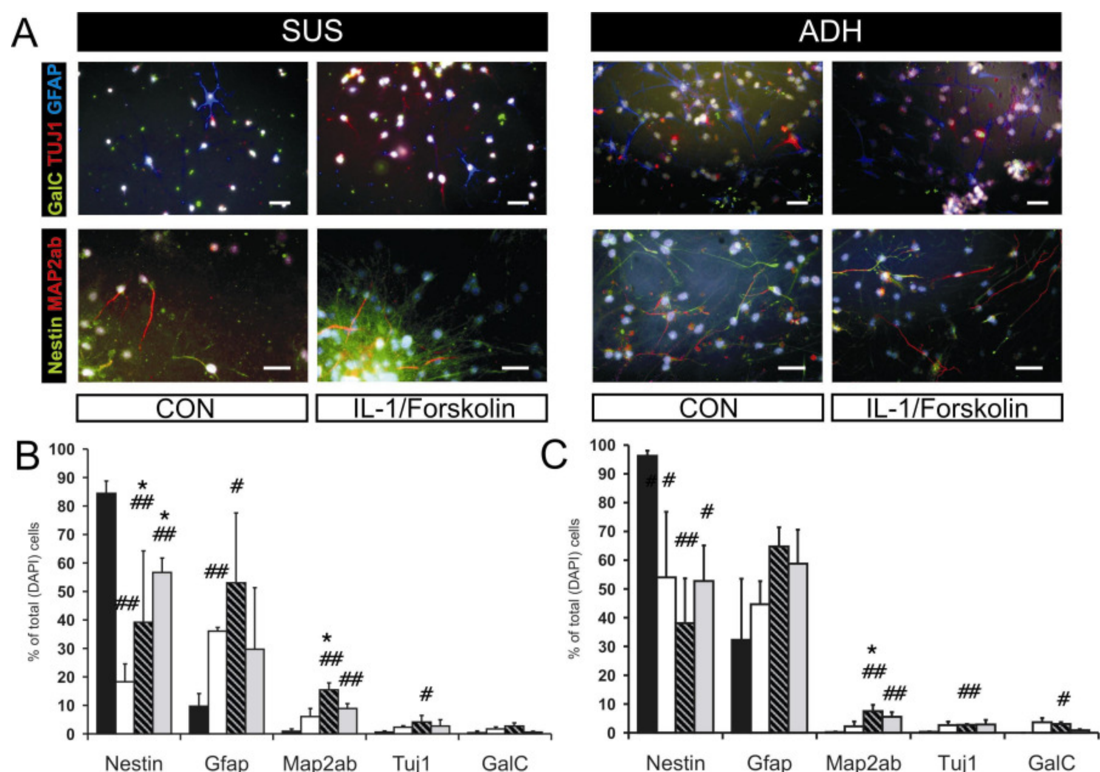


Fig. 9 Differentiation characteristics of E14 fetal mouse NPCs. **A**: Immunocytochemistry of E14 suspension (left panels) and adherent (right panels) NPCs. Scale bar: 50 µm. **B**: Quantitative analysis of immunocytochemistry data of E14 suspension NPCs. NPCs: black, no factor (control): white, IL-1 and Forskolin: diagonal stripes, Fgf-8, Shh and Forskolin: grey. **C**: Quantitative analysis of immunocytochemistry data of E14 adherent NPCs. NPCs: black, no factor (control): white, IL-1 and Forskolin: diagonal stripes, Fgf-8, Shh and Forskolin: grey. Data are expressed as mean ± s.e.m. # - p≤0.05 (to NPCs), ## - p≤ 0.01 (to NPCs), * - p≤0.05 (to control), ** - p≤ 0.01 (to control).

differentiation of suspension culture NPCs the amount of Nestin-positive cells was significantly decreased in all samples (**Fig. 9A, B**). The reduction of Nestin expression was most apparent in control samples that underwent mitogen removal without additional factors: from 84±4% in NPCs to 18±6% in control. IL-1/Forskolin treatment resulted in 40±25% Nestin-positive cells, while Fgf-8/Shh/Forskolin resulted in 56±5% Nestin-immunoreactive cells. Gfap immunoreactivity was increased in differentiation control (36±1%) and IL-1/Forskolin (52±25%) compared to NPCs (10±4%). In IL-1/Forskolin treated samples significantly more Map2ab-positive (16±2%) cells were detected compared to NPCs (0.9±0.8%; p≤0.01, n=3) and to control (6±3% and 2±0.6%; p≤0.05, n=3) and significantly more Tuj1-positive (4±2%) cells were detected compared to NPCs (0.6±0.4%; p≤0.05, n=3). That is a 17-fold (Map2ab) and 7-fold (Tuj1) increase compared to NPCs and two-fold to control. Treatment with Fgf-8/Shh/Forskolin resulted in more Map2ab-positive (9±2%; p≤0.01, n=3) cells compared to NPCs (0.9±0.8%). GalC-positive cell numbers did not change significantly in suspension cultures. (**Fig. 9A, B**).

In adherent NPCs Nestin was also detected in significantly fewer cells in all differentiated samples compared to NPCs (**Fig. 9A, C**). Control differentiation conditions yielded 55±22% (p≤0.05, n=3) Nestin-positive cells, IL-1/Forskolin yielded 38±15% (p≤0.01, n=3) and Fgf-8/Shh/Forskolin 53±12% (p≤0.05, n=3) Nestin-positive cells. The number of Gfap immunoreactive cells did not change under differentiation conditions, whereas more GalC cells were present when adherent cells were treated with IL-1/Forskolin (3±0.6 compared to 0±0 in adherent NPCs). IL-1/Forskolin and Fgf-8/Shh/Forskolin were effective for the generation of neurons; the substances yielded 8±2 and 6±2% Map2ab-positive cells respectively. Only the combination of IL-1/Forskolin led to an increase in Tuj1-positive cells (2.7±0.3%; p≤0.01, n=3) compared to NPCs (0.5±0.1%) (**Fig. 9A, C**). Suspension NPCs had a larger neurogenic potential than adherent NPCs. They yielded up to 16±2% Map2ab-positive cells in relation to the total cell number, whereas adherent cells generated a maximum of 9±2% Map2ab-positive cells (**Fig. 9B, C**). All neurons generated from long-term expanded cultures must have emerged *in vitro* because primary

neurons do not survive a long period *in vitro*. This is confirmed by the observation of shorter processes in long-term cultures than usually observed in primary cultures (data not shown).

E10 and E12 fetal mouse NPCs were also examined for their potential to generate differentiated cell types. According to the procedure for E14 NPCs, E12 and E10 NPCs were grown for at least 12 weeks prior to plating onto Poly-D-Lysine cell culture dishes, differentiating with or without factors for at least one week prior to immunocytochemical analysis. Long-term expanded E10 and E12 NPCs were able to generate moderate numbers (5-30%) of neurons (Tuj1) and glia (Gfap (30-70%) and GalC (less than 5% of total cell numbers) just as their E14 counterparts (**Table 4**).

Table 4: Presence of differentiated cell types in differentiated NPCs of ontogenetically differently derived NPCs. +++ Well present in differentiated NPCs (30-70% of total cells), ++ moderately present in differentiated NPCs (5-30% of total cells), + somewhat present in differentiated NPCs (under 5%).

	E14	**E12**	**E10**
Tuj1-positive cells	++	++	++
Gfap-positive cells	+++	+++	+++
GalC-positive cells	+	+	+

In summary, E14, E12, and E10 NPCs generated the major cell types of the central nervous system after long-term expansion. E14 suspension NPCs had a larger neurogenic potential than adherent NPCs. On mRNA level, the neuronal marker *Tuj1* and the dopaminergic marker *Pitx3* were upregulated, whereas *Nr4a2* showed no regulation at the 7d stage during differentiation of E14 NPCs.

4.4. Neuron-specific differentiation of fetal mesencephalic NPCs.

For the further characterization of the neurogenic potential of long-term expanded NPCs the progenitor cultures were differentiated for 7 days and then subjected to immunocytochemistry of different neuronal markers or total RNA was isolated and qRT-PCR was performed for *Th* mRNA. Suspension NPCs did not transcribe significantly more *Th* mRNA compared to NPCs when differentiated (**Fig. 10A**). Adherent fetal NPCs generated *Th* mRNA when differentiated with IL-1/Forskolin (0.006±0.001; p≤0.01, n=3) in

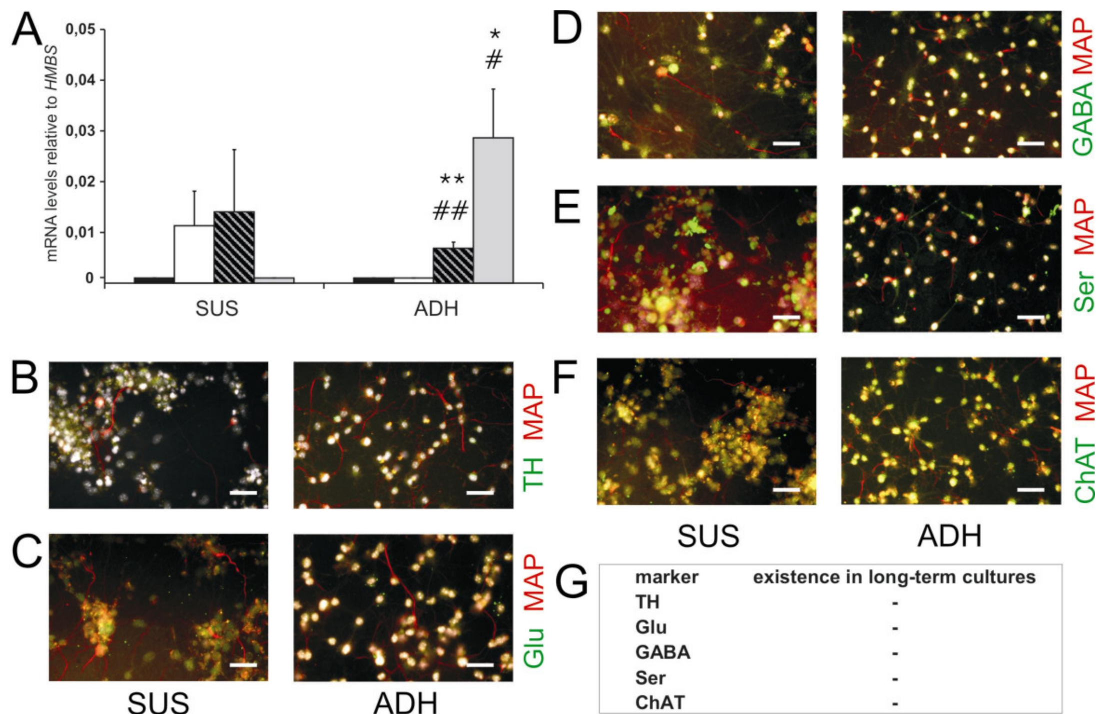


Fig. 10 Characteristics of differentiated E14 fetal mouse NPCs. **A**: Q (RT) PCR of *Th* mRNA in suspension (left) and adherent (right) culture. Differentiated samples (no factor: white, IL-1 and Forskolin: stripes, Fgf-8, Shh and Forskolin: grey) are shown relative to NPCs (black). Data are expressed as mean ± s.e.m. # - p≤0.05 (to NPCs), ## - p≤ 0.01 (to NPCs), * - p≤0.05 (to control), ** - p≤ 0.01 (to control) **B-F**: Immunocytochemistry of E14 suspension (left panels) and adherent (right panels) NPCs. Scale bar: 50 µm. **G**: Summary of markers present in long-term NPC cultures.

comparison to control (=0) and with Fgf-8/Shh/Forskolin (0.028±0.009; p≤0.05, n=3) in comparison to control (=0) as well as NPCs (=0) (**Fig. 10A**).
E14 fetal mesencephalic NPCs did not have the potential to differentiate into dopaminergic, glutamatergic, GABAergic, serotonergic or cholinergic neurons after long-term cultivation (**Fig. 10B-G**). Neither suspension nor adherent culture NPCs showed positive stains for the neuronal markers suggesting that long-term proliferated fetal mouse NPC cultures did not have the potential to differentiate into neuronal subtypes. That could have been due to a relatively short differentiation procedure (7 days) that resulted in immature neurons, or the problem was the long expansion time *in vitro*. However many long-term proliferated and differentiated cells were positive for Map2ab, demonstrating a rather mature cell population after differentiation. Surprisingly, NPCs from E12 and E10 failed to differentiate into dopaminergic neurons just as their counterparts from E14 (data not shown). This finding suggested that the failure of E14 NPCs to generate dopaminergic neurons was not a developmental distinctiveness of NPCs.

4.5. Short-term expansion of fetal mesencephalic NPCs.

To determine whether fetal mouse NPCs lose their differentiation potential over long periods *in vitro*, E14 suspension NPCs were short-term proliferated (two weeks), subsequently differentiated and analyzed by immunocytochemistry. Fetal mouse NPCs displayed dopaminergic neurons (Th-positive) after short-term expansion and subsequent differentiation (**Fig. 11A, B**). NPCs differentiated with IL-1/Forskolin (0.53±0.12%; p≤0.05, n=3) yielded more dopaminergic neurons than control (no factor differentiated) samples (0.22±0.06%) (**Fig. 11A, B**). Fgf-8/Shh/Forskolin treatment did not lead to significantly more dopaminergic neurons than in control conditions. IL-1/Forskolin samples (12.2±2.1%; p≤0.01, n=3) developed more Map2ab positive cells than control samples (3.2±0.9%) and stained for less progenitor (Nestin-positive) cells (22.8±5.6%; p≤0.01, n=3) than control samples (66.0±3.8%), whereas Fgf-8/Shh/Forskolin samples contained the

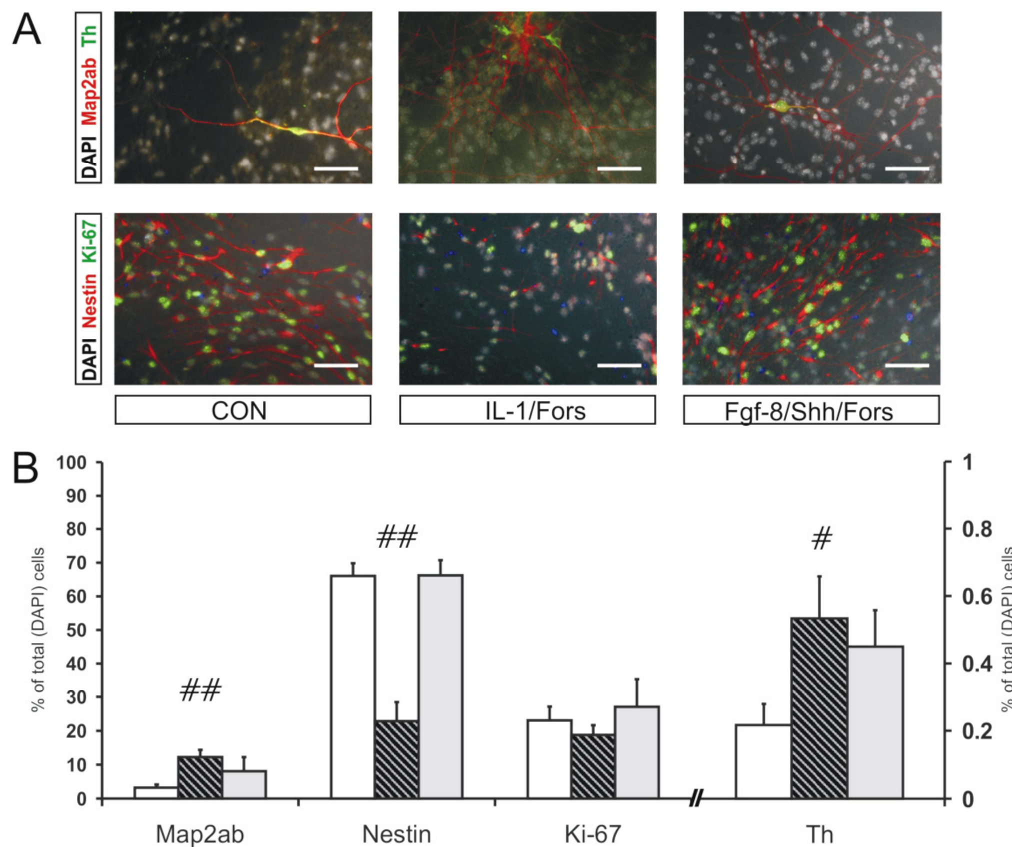


Fig. 11 Characteristics of differentiated fetal (short-term expanded) NPCs. **A, B**: Immunocytochemistry of E14 suspension NPCs differentiated with no factor (white), IL-1 and Forskolin (diagonal stripes), Fgf-8, Shh and Forskolin (grey). Data are expressed as mean ± s.e.m. # - p≤0.05, ## - p≤ 0.01 (to control). Scale bar: 50 µm.

same amounts of Map2ab (8.0±4.1%) and Nestin (66.0±4.5%) positive cells as control samples. The proliferative marker Ki-67 was present in 20-30% of total of cells, irrespective of the experimental condition (**Fig. 11A, B**).
Short-term cultivation before differentiation did not allow excluding carry-over of primary neurons from the isolation process, therefore other methods to demonstrate the *in vitro* generation of differentiated cell types were necessary.

To show that the generated neurons and glial cells were *de novo* generated from proliferating cells, NPCs were labeled with BrdU immediately before differentiation (**Fig. 12**). NPCs were expanded for two weeks, incubated with 10 µM BrdU for 24 h to label all dividing cells, washed, allowed to regenerate in fresh medium and then differentiated. After one week of differentiation, the samples were immunocytochemically processed.

Rarely, oligodendrocytes (**Fig. 12A**) and astrocytes (**Fig. 12B**) were co-labeled with BrdU, suggesting a cell division immediately before terminal differentiation. Dopaminergic neurons, glutamatergic neurons and GABAergic neurons were observed in short-term expanded NPCs (**Fig. 12C,**

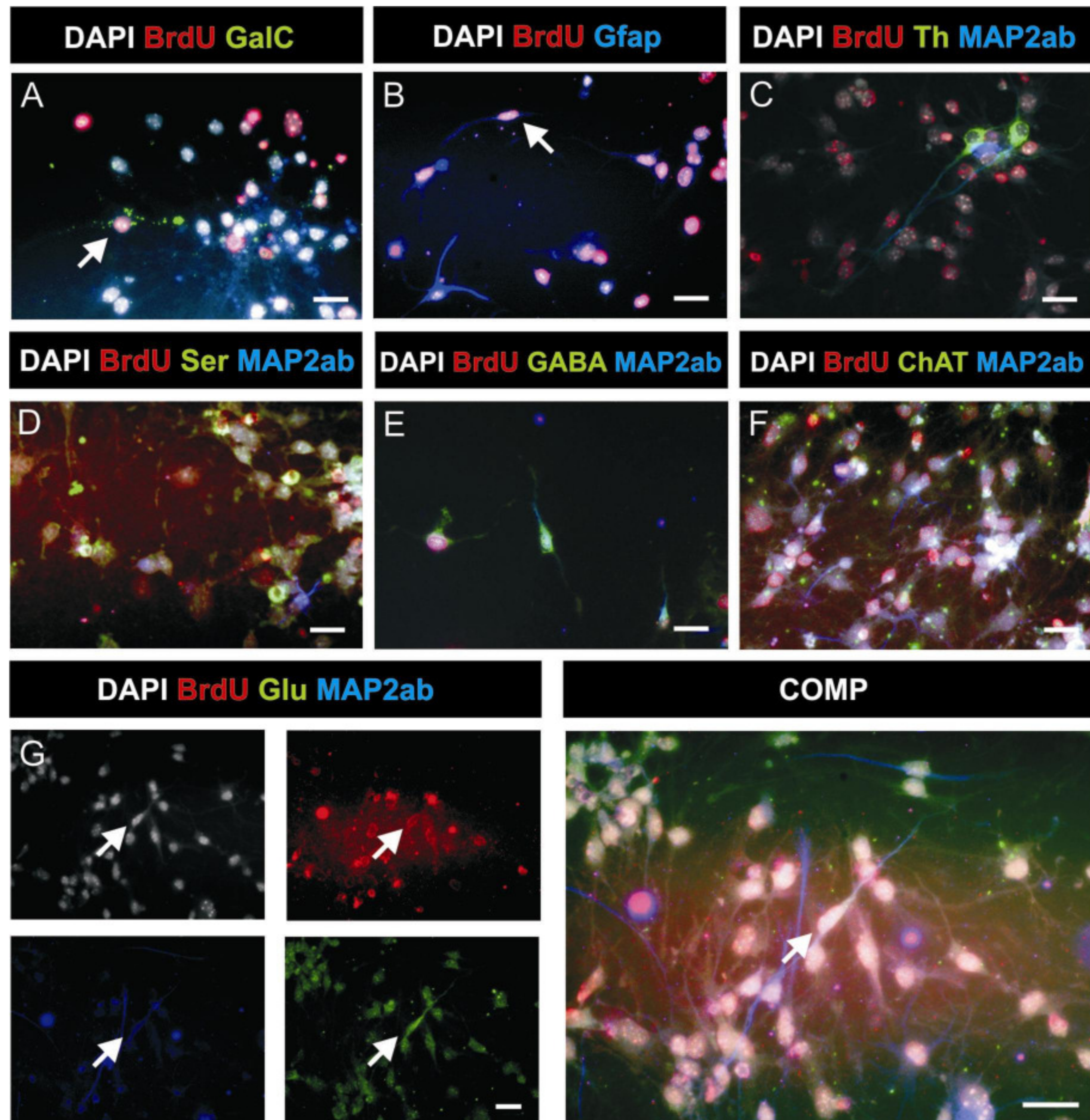


Fig. 12 Characteristics of differentiated fetal (short-term expanded) NPCs. **A-G**: Immunocytochemistry of E14 suspension NPCs that were labeled with BrdU and subsequently differentiated with no factor or IL-1 and Forskolin. Oligodendrocytes (GalC-positive) and Astrocytes (Gfap-positive) were co-labeled with BrdU (arrows in **A** and **B**). Dopaminergic (Th-positive, **C**), Serotonergic (Ser-positive; **D**), GABAergic (GABA-positive; **E**) or Cholinergic (ChAT-positive; **F**) neurons were not co-labeled with BrdU. Glutamatergic (Glu-positive; **G**) neurons were rarely co-labeled with BrdU. Scale bar: 25 µm.

E, G). No dopaminergic or GABAergic neuron also stained for BrdU (**Fig. 12C, E**). However, glutamatergic (**Fig. 12G**) neurons were observed in the culture and sometimes also stained for BrdU. Not present in short-term

expanded NPCs from fetal midbrain were serotonergic and cholinergic neurons (**Fig. 12D, F**).

These observations suggested that progenitors from the midbrain were regionally committed and were not able to form serotonergic or cholinergic neurons. Glutamatergic and GABAergic neurons were present in short NPC cultures, glutamatergic neurons being newly formed *in vitro* after cell division of precursor cells. Because they failed to incorporate BrdU, GABAergic and dopaminergic neurons could not have been formed before or at early time-points during differentiation. Presumably, cell division took place later in the differentiation phase, or not at all, suggesting terminal differentiation from committed precursor cells without cell division. Another possibility was that primary neurons carried over from the isolation process contaminated the experiments. BrdU-labeling during differentiation did not work because all neurons died during that process. It was therefore impossible to rule out formation of new neurons by cell division in the differentiation phase, yet this was not probable because cell divisions decreased rapidly during differentiation. To exclude carry-over of neurons from the isolation process, Th-Gfp mice were used. These mice carried Gfp in the tyrosine hydroxylase locus, thus all dopaminergic neurons appear green. After isolation of fetal mesencephalic tissue (E14), the cells were FACS-sorted, removing all Th-Gfp-positive cells (primary dopaminergic neurons) from the cell suspension (**Fig. 13**). During the sorting, dead cells were excluded by forward scatter analysis, and Gfp-positive cells were isolated from Gfp-negative cells (**Fig. 13A**). The Gfp-negative fraction contained 0.1% Gfp-positive cells (green frame; **Fig. 13B**). After this process the Th-Gfp negative fraction and a mixed NPC fraction were cultivated for one week, subsequently differentiated and analyzed by immunocytochemistry. In the mixed fraction the formation of dopaminergic neurons was evident (1.22% of total cell number) (**Fig. 13C, D**). Interestingly, in the Th-Gfp-negative fraction, also dopaminergic neurons were genereated (1.9% of total cell number) - more than contaminations of FACS sorting would amount to (0.1% of total cell number) (**Fig. 13C, D**). This ruled out the observation of contaminating primary neurons in short-term expanded NPCs. Instead, dopaminergic neurons must have formed

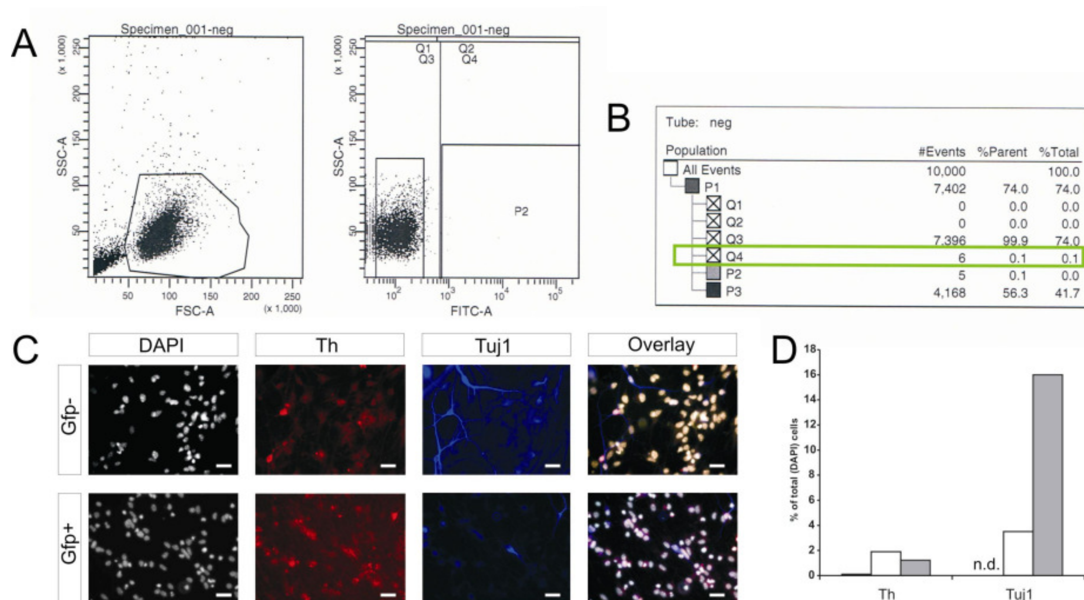


Fig. 13 Characterization of short-term expanded NPCs. **A, B**: FACS-Sorting of isolated Th-Gfp fetal mesencephalic NPCs. Th-Gfp negative fraction (Q4) contained 0.1% Th-Gfp-positive cells (green frame). **C**: Immunocytochemistry of differentiated E14 NPCs from the Th-Gfp-negative fraction. Scale bar: 25 µm. D: Quantitative analysis of FACS sorted, proliferated, differentiated NPCs: Contaminations of primary cells after FACS sorting (black), differentiated Gfp-negative fraction (white), differentiated mixed fraction (grey).

in vitro. In summary, Th-positive (dopaminergic) neurons were observed in short-term culture of fetal mouse NPCs, but they were not formed through cell division prior to or at early time-points of differentiation. Presumably, they formed from committed neural precursors without cell division. Additionally to Th-positive neurons, GABAergic and glutamatergic neurons were observed in short-term cultures. Only glutamatergic neurons formed through cell division. However, mouse fetal midbrain NPCs generated oligodendrocytes (GalC) and astrocytes (Gfap) through cell division of progenitor cells.

4.6. Comparison of short-term and long-term expanded NPCs.

The failure of long-term expanded precursors to generate dopaminergic neurons on one hand and the existence of precursors that form dopaminergic neurons in the short-term expanded NPC culture on the other hand lead to the analysis of markers in NPC cultures at different stages of *in vitro* cultivation (**Fig. 14**). Total RNA was isolated from different NPC cultures and analyzed by qRT-PCR for the existence of markers thought to be involved in different stages of neuronal precursor development. Short-term expanded NPCs expanded for 14d and 21d only

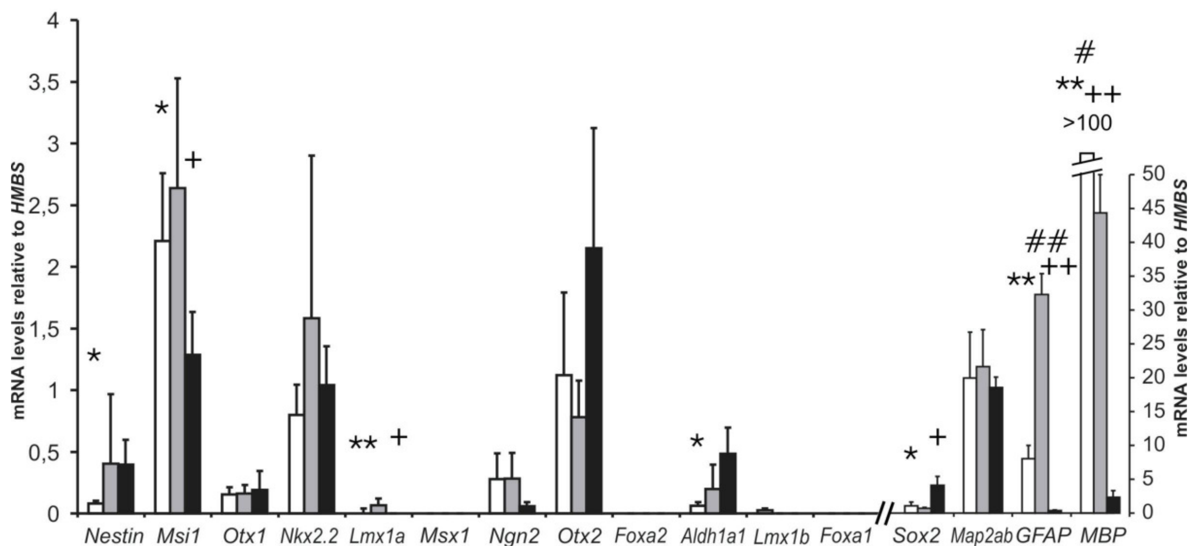


Fig. 14 Characteristics of E14 fetal mouse NPCs. Q (RT) PCR of suspension culture NPCs that were cultivated 14 d *in vitro* (white), 21 d *in vitro* (grey) and more than 80 days *in vitro* (black). Data are expressed as mean ± s.e.m. # - p≤0.05 (14d compared to 21d) , ## - p≤ 0.01 (14d compared to 21d). * - p≤0.05 (14d compared to >80d) , ** - p≤ 0.01 (14d compared to >80d). + - p≤0.05 (21d compared to >80d) , ++ - p≤ 0.01 (21d compared to >80d).

differed in their content of *GFAP* and *MBP* mRNA. *GFAP* mRNA was more abundant in the somewhat older culture (32±3 compared to 8±2 relative to *HMBS*; p≤0.01, n=4), whereas more *MBP* mRNA was determined in the younger *in vitro* cultures (105±17 compared to 44±28 relative to *HMBS*; p≤0.05, n=4) (**Fig. 14**). In comparison to >80 days (>80d) old NPCs short-

term expanded NPCs (14d) contained more mRNA for the stem cell marker *Musashi* (*Msi*; 2.2±0.5 compared to 1.3±0.3; $p \leq 0.05$, n=4)), and the glial markers *GFAP* (8±2 compared to 0.36±0.11 relative to *HMBS*; $p \leq 0.01$, n=4) and *MBP* (105±17 compared to 1.1±0.9; $p \leq 0.01$, n=4). On the other hand, mRNA for stem cell markers *Nestin* (0.39±0.2 compared to 0.08±0.02 relative to *HMBS*; $p \leq 0.05$, n=4), *Sox-2* (4.1±1.3 compared to 1.0±0.5 relative to *HMBS*; $p \leq 0.05$, n=4), and *Aldh1a1* (0.48±0.21 compared to 0.06±0.03 relative to *HMBS*; $p \leq 0.05$, n=4) were more abundant in long-term expanded NPCs. Similarly, NPCs expanded for 21 days (21d) *in vitro* also transcribed more mRNA for the stem cell marker *Msi* (2.64±0.89 compared to 1.3±0.3 relative to *HMBS*; $p \leq 0.05$, n=4) and the glial markers *GFAP* (32±3 compared to 0.36±0.11 relative to *HMBS*; $p \leq 0.01$, n=4) and *MBP* (44±28 compared to 1.1±0.9 relative to *HMBS*; $p \leq 0.01$, n=4) than long-term expanded NPCs. *Sox-2* mRNA was determined in higher quantities in long-term expanded NPCs (4.1±1.3 compared to 0.68±0.16 relative to *HMBS*; $p \leq 0.05$, n=4) (**Fig. 14**). The controversial outcome mirrored the initial heterogeneity and ongoing change of the *in vitro* cultures.

Equally transcribed in the different stage *in vitro* cultures were *Otx1* (neuroepithelial neuroblasts), *Otx2* (VZ/SVZ progenitors), *Nkx2.2* (neuroepithelial neuroblasts), *Ngn2* (VZ/SVZ progenitors) and *Map2ab* (neurons). Not transcribed at all stages were *Msx1* and *FoxA1* (ventral midbrain dopaminergic precursors) as well as *FoxA2* (VZ/SVZ progenitors). *Lmx1b* (ventral midbrain dopaminergic precursors) was only transcribed in very early (14d) *in vitro* cultures (0.02±0.01 relative to *HMBS*), and *Lmx1a* (VZ/SVZ progenitors) was only transcribed in both early cultures (0.10±0.04 and 0.07±0.05 relative to *HMBS*), not in long-term expanded NPCs.

Combined, these data showed that a diversity of changes were going on and overlapping one another in *in vitro* NPC cultures. Importantly, markers of differentiated cell types (*GFAP, MBP, Map2ab*) stayed the same or went down, and markers for ventral midbrain dopaminergic precursors (*FoxA1, Lmx1b*) were absent or became absent over the course of NPC culture, suggesting an 'aging' effect of *in vitro* conditions. Markers for VZ/SVZ progenitors or neuroepithelial neuroblasts were transcribed variably in differently aged NPCs.

4.7. Existence of IL-1RI and IL-1 **in vitro** ***and*** **in vivo** ***at E 14.***

Interleukin (IL)-1 is a thoroughly investigated inflammatory cytokine involved in cellular activation and cell-to-cell communication. Besides its function in the immune system, a potential role as neuromodulator in the normal brain is emerging (181). It was shown that IL-1 is a potent inducer of a dopaminergic phenotype from fetal mouse as well as rat and human (26,101,136,159,160) mesencephalic precursors *in vitro*. The intracellular signaling cascades activated by IL-1 were p38 and Erk1/2 mediated. To investigate whether the effects were *in vitro* artifacts or whether IL-1 might play a role in the developing brain, the presence of IL-1 isoforms and the dominant and functional receptor IL-1RI *in vitro* and *in vivo* was established.

Long-term suspension NPC cultures were used to detect IL-1α, IL-1β and IL-1RI *in vitro* (**Fig. 15**). Neurospheres were plated onto Poly-D-Lysine

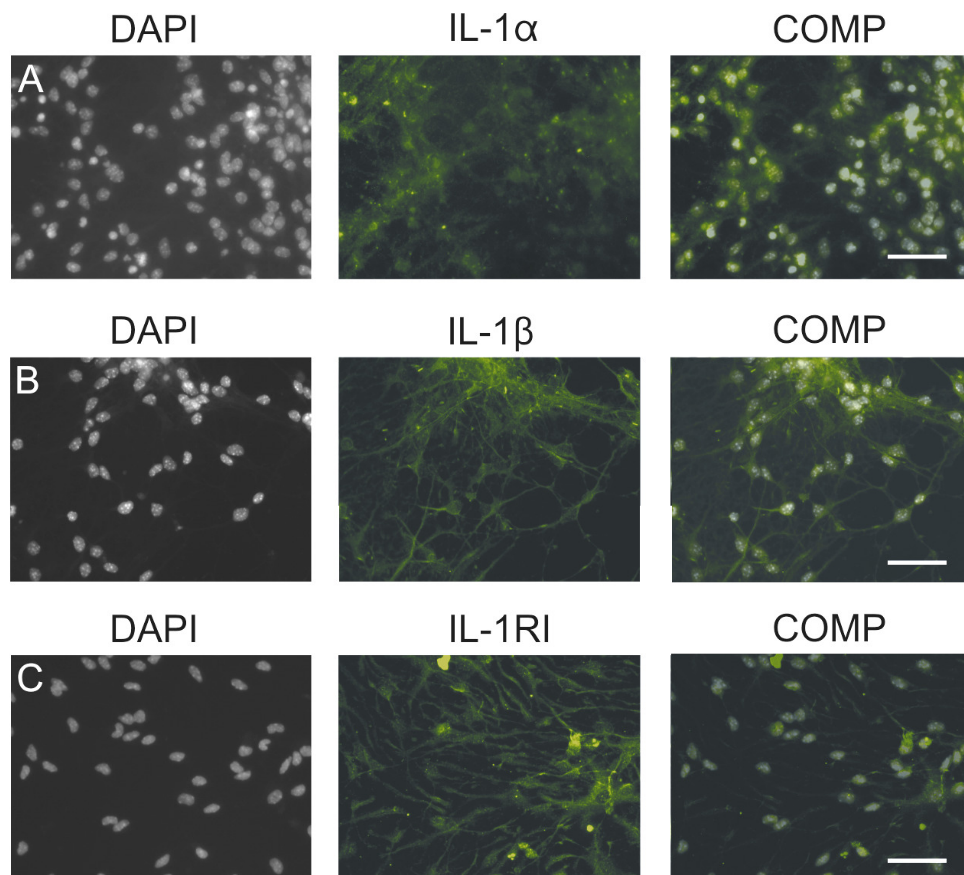


Fig. 15 Immunofluorescence of E14 long-term expanded NPCs for Interleukin (IL)-1α (**A**), IL-1β (**B**) and IL-1 receptor type I (**C**) in green counterstained with DAPI (white). Scale bar: 50 µm.

coated culture dishes and left to adhere for 3h before fixation and subsequent immunofluorescence procedure. IL-1α, IL-1β and IL-1RI immunoreactivity was expected in the cytoplasm or plasma membrane of cells. IL-1α immunoreactivity was detected in all compartments of many but not all cells in long-term NPC culture (**Fig. 15A**). It was therefore assigned to unspecific binding of the antibody and regarded as not expressed. IL-1β immunoreactivity was observed mainly in the cytoplasm of body and processes in most cells (**Fig. 15B**). IL-1RI immunoreactivity was detected in the cytoplasm of the cell body in only few cells (**Fig. 15C**). IL-1β and IL-1RI were expressed in long-term NPC culture. The location of both proteins would have to be specified using confocal microscopy. The existence of a signal (IL-1β) and a transducer (IL-1RI) suggested a possible role in the *in vitro* differentiation of fetal NPCs.

IL-1α, IL-1β, and IL-1RI immunoreactivity *in vivo* was assessed in brains of E14 mouse embryos (**Fig. 16**). Pregnant mice were perfused at E14, embryonic brains were dissected, quick-frozen, sectioned, and delivered to immunohistochemical analysis. Sections from midbrain regions of the brain were used. Images were obtained by classical fluorescence or confocal microscopy. No specific signal was obtained staining with the IL-1α antibody (**Fig. 16A**). It was assumed that no IL-1α immunoreactivity was present in the developing mouse midbrain. IL-1β immunoreactivity was present close to the ventricle in contrast to TUJ1 immunoreactivity, which occurred more laterally (**Fig. 16B**). The antibody for IL1RI apparently stained for vessels lacking other specific staining in the midbrain (**Fig. 16C**). It could be concluded that at least IL-1β was present in the developing mouse midbrain.

In summary, long-term expanded fetal mesencephalic NPCs could be grown under suspension or adherent culture conditions and showed self-renewing capacity as well as markers typical for NPCs. They could be differentiated into the three major cell types of the nervous system, but suspension NPCs had a larger potential to generate neurons than adherently grown NPCs. Signaling cascades involved in this process were activated by IL-1 and were p38 and Erk1/2 mediated. Long-term expanded NPCs did not have the potential to generate any neuronal sub-types. When

cultivated *in vitro* for short periods, fetal mesencephalic NPCs were able to generate dopaminergic neurons that probably did not arise through cell division, but differentiated from committed precursor cells. IL-1 and Forskolin were the most potent inductors of the dopaminergic fate. Glutamatergic

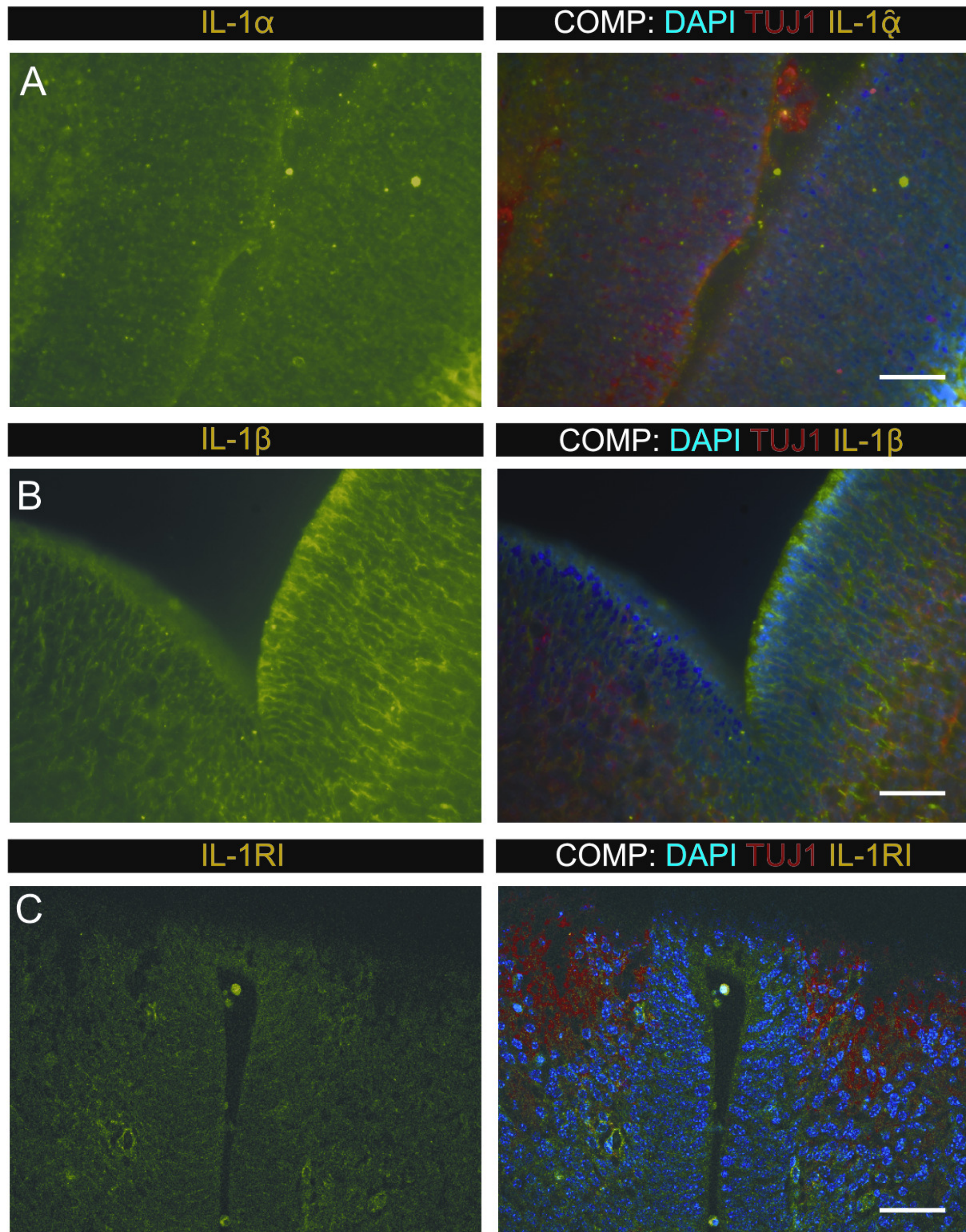


Fig. 16 Immunohistochemical analysis of E14 brains (sagittal sections) for Interleukin (IL)-1α (**A**), IL-1β (**B**) and IL-1 receptor type I (**C**) in green. Sections were counterstained with the neuronal marker Tuj1 (red) and DAPI (blue). Scale bar: 50 µm.

neurons were present in short-time expanded NPC cultures and were shown to be generated through cell division from progenitors. The precursor population of short cultures differed from long- term expanded cultures, suggesting an 'aging' effect of *in vitro* conditions. IL-1β was present inNPCs *in vitro* as well as close to the ventricular zone *in vivo* suggesting an expression in progenitor cells rather than differentiated cells. IL-1RI was expressed only *in vitro,* whereas IL-1α was not expressed *in vivo* or *in vitro*.

5. Discussion

Neural stem (progenitor) cells (NPCs) from fetal tissue have been identified as a rapidly dividing source of cells that are able to generate cells of all three neural lineages and do not divide uncontrolled once transplanted into a host organism.

Fetal mouse NPCs are a crucial tool for investigations on neural stem cell differentiation, because they make the employment of transgenic animals *in vivo* and cells *in vitro* possible. So far only short-term expanded NPC cultures were shown to display dopaminergic neurons and it was not clear whether this was due to real differentiation or a result of increased survival of primary dopaminergic neurons.

The aims of the thesis were to characterize mouse fetal NPCs, to establish the long-term expansion of fetal mouse NPCs and the generation of dopaminergic neurons in long-term expanded fetal mouse NPCs, to investigate the signaling mechanisms involved in the differentiation of mouse fetal NPCs towards the dopaminergic phenotype and to compare NPCs that were cultivated for short periods *in vitro* with long-term expanded NPCs.

5.1. Long-term expanded NPCs

Mouse fetal mesencephalic NPCs were used to describe the self-renewing and differentiation potential of mixed progenitor cultures. Long-term expanded fetal mesencephalic NPCs could be grown under suspension or adherent culture conditions. In contrast to adult NPCs (48,130,131,141,152), fewer cells were needed to generate a neurosphere, showing the higher proliferative capacity of fetal NPCs. However, the neurosphere forming assay depended on the quick execution of the dilution series and was therefore variable in outcome.

Adherently grown NPCs harbored 97% Nestin-positive cells in E14, E12, and E10 NPC cultures. Suspension culture NPCs had a smaller proportion of Nestin-positive cells which was probably due to the three-dimensional shape of spheres. Nestin was the NPC marker of choice

because no better marker was available. However, Nestin is also expressed in different germ layers (154) and in postmitotic neural cells (32), its usefulness as a stem cell marker being therefore limited. Hence, other markers typical for NPCs were analyzed to monitor self-renewing capacity of the cultures such as *Sox-2, Prominin,* and *Musashi1* (*Msi),* which were more abundant in suspension culture cells. This controversy compared to Nestin expression demonstrated that one marker rarely characterizes a cell type appropriately, that a combination of markers will define a true stem or progenitor cell (20). It also stressed that fetal NPC cultures were a heterogeneous source of progenitors as well as differentiated cells, because markers for differentiated neural cells were expressed, albeit at low levels or in few cells.

Suspension NPCs transcribed *Egf-R* in much lower levels than adherent NPCs. Egf-R becomes expressed in development in a subpopulation of progenitors that previously acquired Egf responsiveness (21,87). Interestingly, according to the developmental change, Egf-R expression occurred on schedule (96) in suspension cultures (starting at E14) but not in adherent cultures, suggesting that cell-to-cell signaling is required. Since the higher transcription rate of *Egf-R* mRNA in adherent NPCs was observed from E10 (mostly Fgf-responsive cells) to E14 (Fgf- and Egf-responsive cells), the addition of both Fgf-2 and Egf may have lead to a higher Egf-R expression in adherent NPCs because regulatory elements may also require cell-to-cell contact.

E14 NPCs were analyzed for the transcription of several markers known to be expressed in different populations of midbrain progenitors (20,157). Of neuroepithelial neuroblast markers both Otx1 and Nkx2.2 were present. Ventricular zone (VZ)/subventricular zone (SVZ) progenitor markers Otx2, Aldh1a1, and Ngn2 were present; Lmx1a and FoxA2 were not transcribed in E14 NPCs. Markers for ventral midbrain dopaminergic precursors were present in part: En1 and En2 were transcribed. On the other hand Msx1, Lmx1b, FoxA1, Nr4a2, and Pitx3 were not transcribed. This transcription profile suggested that a mixed population of progenitors was cultivated *in vitro*. It was also a hint that either certain markers for temporal and spatial origin were lost while others were maintained *in vitro*,

that progenitor populations (20) can be described with less markers than previously anticipated or that certain sub-populations of progenitors perish early *in vitro*.

In E14 NPCs the same amount of cells was Ki-67-positive as well as BrdU-positive, when BrdU labeling was done for 20h in E14 NPCs. That meant that the proliferating part (Ki-67-positive) of the culture had doubled (BrdU) in the previous 20h. However, not all BrdU-positive cells were also Ki-67-positive and not all BrdU-positive cells were Ki-67-positive, suggesting that asymmetric cell divisions (known from the neuroepithelium *in vivo*) can also occur *in vitro*, generating one proliferating and one differentiating daughter cell - suggested also by Reynolds and Weiss (142). It must be noted, however, that Ki67 is absent in early G1-phase cells and in cells undergoing DNA repair (51,77). Cells staining for only BrdU therefore could also be proliferating daughter cells (suggesting symmetric divisions) that were residing in the early G1-phase of the cell cycle at the time of fixation.

NPC cultures from different temporal origins (E14, E12, and E10) varied little, all expressed Nestin on one hand and no Th on the other hand. Most strikingly, E10 NPCs transcribed more *Prominin* mRNA than E12 and E14 NPCs, which was feasible since Prominin is regarded as a neuroepithelial stem cell marker (134). Therefore, the E10 NPC culture differed in an important characteristic from E12 and E14 NPCs, and one could conclude that at least part of the temporal identity was maintained *in vitro,* which is supported by Kim et al. who assumed as much using fetal human NPCs (79).

E14 NPCs that were expanded for at least 8-12 weeks *in vitro* to obtain large quantities of cells for cell replacement strategies and to overcome contaminations of primary cells were successfully differentiated into the three major cell types of the nervous system – neurons, astroglia, and oligodendroglia. At an early stage of differentiation the transcription of *Th* mRNA was downregulated, whereas *Pitx3* and *Nr4a2* mRNAs were upregulated, when differentiation was induced by Interleukin- (IL-) 1 and Forskolin, factors known to stimulate *in vitro* dopaminergic specification (26,101,136,160). At the same time phosphorylation of the signaling molecules Erk1/2 and p38 was increased compared to control conditions.

The activation of those signaling cascades was an early answer to the exposition to IL-1 and/or Forskolin and might be involved in the dopaminergic specification of fetal mouse NPCs. Interestingly, factors known to be involved in the development of the midbrain (Shh and Fgf-8) did not lead to an increase of *Pitx3* and *Nr4a2* mRNA nor an increase in p38 phosphorylation. Shh and Fgf-8 have been controversially discussed for their usefulness in *in vitro* differentiation of tissue-specific NPCs, and it might be the case that especially E14 NPCs are ontogenetically too old to be influenced by them (165), whereas ES cell-derived NPCs were successfully driven to adopt a dopaminergic phenotype (91).

At a late stage (7d) of differentiation *Nestin* mRNA was mainly downregulated (significantly in control and IL-1/Forskolin treated adherent as well as all suspension cultures) compared to NPCs. On the protein level Nestin was expressed in fewer cells under all differentiation conditions compared to NPCs in suspension as well as adherent cultures and at the same time differentiation markers were expressed in more cells under differentiation conditions compared to NPCs. These results emphasized that the cultures responded to the removal of mitogens and plating on Poly-D-Lysine with differentiation. The addition of differentiation cytokines or growth factors only intensified the effect.

However, neuronal cell numbers were additionally regulated by incubation with cytokines or growth factors. Map2ab (mature neurons) cell numbers were higher in both culture systems when IL-1/Forskolin or Fgf-8/ Shh/ Forskolin were added compared to NPCs. More cells became Tuj1-positive (immature neurons) when IL-1 and Forskolin were added in suspension as well as adherent cell cultures. Interestingly, suspension NPCs had a larger potential to generate neurons than adherent NPCs. A seemingly high number of progenitors in the NPC cultures (adherent) did not correlate with a high amount of neurons after one week of differentiation (suspension). These results suggested that the microenvironment of neurospheres contributed to the ability of NPCs to generate neurons. Schofield, who invented the ‘niche’ hypothesis, argued that stem cells only retained their pluripotency within an appropriate environment (151).

Long-term expanded NPCs did not have the potential to generate neuronal subtypes; most importantly, they did not generate dopaminergic neurons. Apparently, they differentiated into neurons but stayed in a postmitotic but uncommitted state. Because dopaminergic neurons *in vivo* arise at E10.5, potentially more adequate cell sources were isolated at E12 and E10, but also failed to adopt dopaminergic phenotypes after long-term expansion *in vitro*. This was not resulting from short differentiation periods, since most neurons were Map2ab-positive (mature neurons) rather than Tuj1-positive (immature neurons). The loss of neurogenic potential in fetal mouse NPCs after long-term cultivation was species-specific, since neuronal subtype differentiation *in vitro* had been achieved in rat and human long-term expanded progenitor cultures (26,101,107,136,159,160). Differences in proliferation and differentiation characteristics of NPCs of different species have already been reported previously (158,172).

5.2. Short-term expanded NPCs

When cultivated *in vitro* for short periods, fetal mesencephalic NPC cultures displayed dopaminergic neurons. The amount of Th-/Map2ab-positive cells was significantly higher in IL-1/Forskolin treated cultures compared to control (no factors), but not in Fgf-8/ Shh/ Forskolin treated samples compared to control. Also, Map2ab-positive cells were more frequent in IL-1/Forskolin treated samples compared to control but not in Fgf-8/ Shh/ Forskolin treated samples in short-term expanded, differentiated NPCs. These findings support a previously established role for IL-1 in the dopaminergic specification of fetal NPCs (26,101,107,136,159,160). Compared to long-term expanded cultures, Map2ab-positive cell numbers in short-term cultures were lower due to a lower total cell number because of the shorter expansion time prior to differentiation, resulting in a lower cell density. It must be pointed out that the cell density of cultures has an extraordinarily strong effect on the presence of differentiation markers. An optimal cell density resulted in an overall healthier phenotype and more differentiated cells. It remained unknown whether these mechanisms

influenced all cell types in the culture or whether they influenced glia, which in turn secreted protective substances that supported neuronal cell types.

The initial long-term expansion of NPC cultures was performed to ensure that primary neurons that contaminate early NPC cultures died or were highly diluted before the differentiation process began. In short-term expanded NPC cultures it was impossible to prevent that instead of differentiation the main process leading to a larger number of Th-positive neurons was neuronal protection. To monitor the formation of new neurons *in vitro*, short-term expanded NPC cultures were BrdU-labeled for 24h to include as many cells as possible into the analysis. No Th-/ Map2ab-positive cell was ever also BrdU-positive, suggesting that all dopaminergic neurons found had formed early during the *in vitro* cultivation, had formed without cell division from precursors or were surviving neurons from the midbrain isolation. It was unlikely that they had formed from a reliably expandable source of progenitors, because when cells are BrdU labeled and then divide, the dye is distributed to the daughter cells and they have a less intensive but visible stain, which could have been observed in Th+ neurons. It could not be ruled out though that the dopaminergic neurons formed during the differentiation process, because labeling during differentiation did not work since all cells died during that process. However, this was unlikely, because the removal of mitogens, plating onto Poly-D-Lysine and the addition of IL-1/Forskolin resulted in a strong differentiation signal diminishing the amount of Nestin-positive cells and leading to a much lower amount of cells actively running the cell cycle.

Differentiation after short-term expansion very rarely also lead to GABAergic and glutamatergic subtypes of neurons. In contrast to dopaminergic and GABAergic neurons which were not co-labeled with BrdU, glutamatergic neurons rarely displayed BrdU incorporation and were thus *de novo* generated.

To find out whether fetal mouse NPCs were able to generate dopaminergic neurons *in vitro*, Th-Gfp mice were used. In this mouse, Gfp was expressed from the *tyrosine hydroxylase* locus and therefore all dopaminergic neurons appeared green. This was used to deplete the cell suspension of all dopaminergic neurons via FACS sorting prior to *in vitro*

cultivation. After subsequent short-time expansion and differentiation Th-positive neurons were detected, proving that fetal mouse NPCs are able to generate dopaminergic neurons *in vitro*. Hence, those dopaminergic neurons formed most likely from precursors present at early stages of *in vitro* cultivation without cell division.

The lack of dopaminergic neurons after long-term expansion therefore had to have been a consequence of the loss of progenitors able to generate dopaminergic neurons. It was not clear which factors were missing *in vitro* that would sustain those progenitors. Therefore, short and long-term NPC cultures were compared on the mRNA level for markers important for different precursor populations. As expected, precursor populations that were cultivated *in vitro* for two or three weeks differed from long-term expanded cultures. Markers of differentiated cell types stayed the same or went down and markers for ventral midbrain dopaminergic precursors that were tested were absent or became absent over the course of NPC culture, suggesting an 'aging' effect of *in vitro* conditions. There were a small number of genes that had a notable progress of transcription. *MBP* transcription is highest in very short expanded NPCs and lowest in long-term expanded NPCs. *Lmx1a* and *Lmx1b* are only transcribed at the two or one earliest stage(s) respectively. The markers resembling best the gradual loss of multilineage potential of *in vitro* NPCs are *Musashi-1*, (being highest in short-term cultures, decreasing in long-term suspension cultures and being lowest in adherent NPCs) and *Nestin* (antiproportional; its transcription goes up over cultivation time and increases over long-term suspension culture to adherent culture). These results further the doubts about Nestin alone being a sufficient stem cell marker, and at the same time put forward another known stem cell marker (Musashi-1) that might be better suited to describe multipotential fetal mouse NPCs. The most straightforward explanation for these observations would be that already after 14 days *in vitro* some markers were expressed abnormally. The data should be completed by a comparison with RNA from fetal midbrain tissue to find out if this expression profile was a result of *in vitro* artifacts. However, contributing to these findings was also the isolation of whole midbrain as a source for the stem cell cultures investigated here, leading to the cultivation of a heterogeneous

progenitor culture. Alternatively, these results account for a diversity of changes overlapping one another in *in vitro* NPCs or may also suggest that markers for different subpopulations of progenitors described in the literature are still not reliably identifying all progenitors.

Another known factor for describing the potential of NPCs to self-renew on one hand and differentiate on the other hand is the epidermal growth factor- receptor (Egf-R). Egf-R activity is associated with proliferation and survival of neural stem cells (10) and loss of Egf-R results in a failure of neuroepithelial precursors to differentiate, resulting in a severe reduction of the medial brain in the fruitfly (34). Additionally, it was shown that Fgf-2, but not Egf, supported Calcium-dependent self-renewal of NPCs and early expansion of lineage-restricted progenitors, whereas both growth factors together permitted the initial commitment of NPCs into neuronal and glial phenotypes (111). These findings might partly account for the failure of NPCs cultivated *in vitro* for long periods of time to generate neuronal subtypes, since they were cultivated with both mitogens. It has also been suggested that human recombinant forms of mitogens used for the proliferation can be more efficiently used by rat and human NPCs, possibly through higher affinities between ligand and receptor (158).

The inflammatory cytokine IL-1 was shown to be an efficient inductor of the dopaminergic phenotype, therefore its expression and the expression of its major receptor was analyzed *in vitro* as well as *in vivo* to find out if cytokines might play a role in the normal, developing brain. IL-1β was present in NPCs *in vitro* as well as close to the ventricular zone *in vivo* suggesting an expression in progenitor cells rather than differentiated cells. IL-1RI was expressed only *in vitro*, whereas IL-1α was not shown to be expressed *in vivo* or *in vitro*. IL-1α is an isoform of IL-1β that binds the same receptors with lower affinity. It is thought to be an autocrine rather than paracrine effector and is generally studied in less detail (181). Both, IL-1α and IL-1β have been shown to equally induce a dopaminergic phenotype in *in vitro* cultures of fetal mesencephalic origin (26,101,136,160). Since only IL-1β was shown to be expressed *in vivo*, only IL-1β might have a function *in vivo*, with the transducing receptor unknown. The three-dimensional structure of the members of the human IL-1 family is composed of 12-14 β –

strands (54,138,180). This β-barrel structure is closely related to that of fibroblast growth factor (121), which possesses some IL-1-like activities. This surprising relationship suggested that IL-1 might alternatively exert its function through one of the Fgf-receptors.

6. Conclusions and future prospects

Several important conclusions concerning fetal mouse stem cell behavior could be drawn from the results of this work:

Firstly, the results showed for the first time that in fetal mouse mesencephalic NPCs dopaminergic neurons differentiate from precursors (possibly ventral midbrain dopaminergic (DA) precursors; represented by orange circles) without cell division, therefore consuming those progenitors (**Fig. 17**). Therein fetal mouse NPCs differ significantly from rat and human NPCs or respond differently to the same *in vitro* conditions. Cultivation conditions need to be optimized for fetal mouse NPCs, which has been previously suggested (158,172). The differentiated cells (blue circles, **Fig. 17**) die after some time *in vitro*, contributing to apoptotic cells and cell debris in the core of neurospheres also described by Svenden et al. (172).

Secondly, less committed precursors (for example neuroepithelial or VZ/SVZ precursors, represented by white circles in **Fig. 17**) find

Ventral midbrain DA precursor

Other proliferating precursors

Differentiated or dead cells

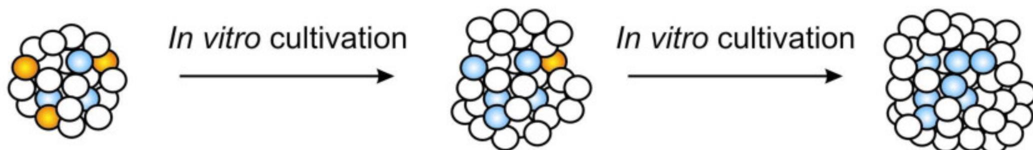


Fig. 17 Schematic representation of the conclusions of this study. A neurosphere generated from fetal mouse midbrain NPCs consists of at least three cell types, ventral midbrain dopaminergic (DA) precursors (orange circles), other proliferating precursors (white circles) and differentiated or dead cells (blue circles) in the core of neurospheres. During the course of *in vitro* expansion, ventral midbrain DA precursors give rise to differentiated cells without cell division, thereby consuming this cell type. Other precursors proliferate but are not able to differentiate under the *in vitro* conditions tested. Differentiated cells eventually die.

appropriate conditions to proliferate but not to generate the more committed DA precursors that are able to generate dopaminergic neurons (possibly due to the effect of Egf and Fgf-2 (111)).

Finally, the hallmarks of stem cells, self-renewal and multipotentiality, seem to be part of a delicate balance, that, when unsettled, goes in favor of one side without the possibility of returning to the previous status. The 'niche' effect in neurospheres results in a better multipotentiality compared to adherent cultures, however, even in neurospheres the *in vitro* cultivation rapidly leads to the loss of dopaminergic potential.

The results obtained in this project helped answer the questions: Can dopaminergic neurons be generated *de novo* from fetal mouse NPCs *in vitro*? Which signaling pathways/molecules are involved in the dopaminergic specification of fetal mouse NPCs? Do short-term and long-term NPCs differ from each other?

Dopaminergic neurons can be generated *de novo* from fetal mouse NPCs *in vitro*. This was proven by eliminating all primary dopaminergic neurons from fetal midbrain cell suspensions prior to *in vitro* cultivation by FACS sorting of midbrain cell suspensions of Th-Gfp mice. Th-positive neurons developed also in the Th-Gfp negative fraction which was depleted of dopaminergic neurons. Other groups have failed to generate (dopaminergic) neurons from fetal mouse NPCs (31,83). It can be hypothesized that this was due to cultivation at atmospheric oxygen levels, because all other conditions were equal to those used here and cultivation at lowered (3%) oxygen conditions had been shown to be useful or necessary for the successful expansion and dopaminergic differentiation of rat and human NPCs (159,160,164,169).

Erk 1/2 and p38 signaling pathways are probably involved in the dopaminergic specification of fetal mouse NPCs. They were activated in long-term expanded NPCs, which were not able to differentiate into dopaminergic neurons. However, these signaling cascades were activated immediately after the differentiation initiation by IL-1/Forskolin and it was shown that IL-1 and Forskolin were potent inductors of the dopaminergic phenotype in other cell types (26,74,101,160). This suggested that Erk 1/2 and p38 were activated in order to differentiate towards a dopaminergic

phenotype but the cascade did not complete and the cells failed to adopt the dopaminergic phenotype. These experiments need to be repeated using short-term proliferated NPCs that are able to generate dopaminergic neurons to prove that Erk 1/2 and p38 are activated during the dopaminergic specification.

Short-term and long-term NPCs differ significantly from each other suggesting an 'aging' effect of *in vitro* conditions. Importantly, markers of differentiated cell types (*GFAP, MBP, Map2ab*) stayed the same or went down from short-term to long-term expanded NPCs, and markers for ventral midbrain dopaminergic precursors (*FoxA1, Lmx1b*) were absent or became absent over the course of NPC culture. Two markers described the gradual loss of multilineage potential of *in vitro* NPCs: *Musashi-1* (highest in short-term cultures, decreasing over long-term suspension cultures to long-term adherent NPCs) and *Nestin* (antiproportional; its transcription goes up over cultivation time and increases furthermore from long-term suspension to adherent culture). These findings suggest that Musashi is a better marker for describing the multipotentiality of fetal mouse NPCs than Nestin. These results need to be confirmed on the protein level. The astrocytespecific glutamate transporter (GLAST) and brain-lipid-binding protein (BLBP) are markers that define radial glial cells - an embryonic cell type able to self-renew and differentiate *in vivo*. It would be valuable to determine their expression in short as well as long-term NPCs to find out if they are also suitable to describe multipotential progenitors *in vitro* and to find out which marker expression must be sustained to support the multipotential capacity of fetal NPCs *in vitro*.

The results of this project lead to the hypothesis that different brain size of an organism results in different margins for stem cell amplification. Fetal mouse NPCs therefore had a smaller margin in which they divide to produce proliferating offspring. This implicates that stem cells have an intrinsic timer for how long they generate progenitors and when they adopt more committed fates themselves and that they keep these cell-intrinsic information *in vitro*. It would be important to find out how to reconstruct this intrinsic memory towards a self-renewing and multipotential mouse fetal progenitor cell *in vitro*.

Further research should focus on two coherent issues: the isolation of more pure populations of progenitors and the more precise characterization of progenitor populations to find out which *in vitro* conditions need to be provided to keep the balance between proliferation and differentiation potential. The knowledge gained about stem cells this way would help establish cell sources for transplantation strategies. In practical terms, smaller parts of the developing midbrain should be isolated and analyzed for markers of progenitor cells before and during the course of *in vitro* expansion. Other growth factors should be added during the *in vitro* expansion, and/or mouse recombinant Egf and Fgf-2 should be obtained.

Conditioned medium from suspension culture NPCs should be added to adherent NPCs to find out whether the higher neurogenic potential of suspension NPCs was a result of the microenvironment in neurospheres. Subsequently, factors that are responsible for this higher neurogenic potential in suspension NPCs should be identified. Conditioned media could also be employed to find out wether there is yet a way to stimulate long-term expanded NPCs to differentiate into dopaminergic neurons.

At long sight, improved fetal mouse NPC cultures and their differentiated counterparts must be transplanted into parkinsonian animals to improve these NPCs as a transplantable cell source.

Additionally, a possible signal transducer for IL-1 *in vivo* should be traced to further the knowledge on this effective dopaminergic agent.

Long-term expansion of isolated fetal precursor cells is necessary to obtain cell numbers sufficient for transplantation strategies. Since long-term expanded NPCs were not able to differentiate into dopaminergic neurons, it is also necessary to discuss other ways to obtain NPC cultures with the potential to become dopaminergic neurons in sufficient quantities. One way that is starting to be discussed, is epigenetic reprogramming of more restricted neural progenitors into NPCs that are easily expanded and have a wider differentiation potential (37,68,80).

The ultimate goal should be to establish a safe and reliably expandable cell source without tumorigenic potential that is able to differentiate into dopaminergic neurons and supporting glial cells. Such a

cell source would improve and support efforts at transplantion strategies for neurodegenerative diseases.

7. References

1 Akiyama, Y., Honmou, O., Kato, T., Uede, T., Hashi, K. and Kocsis, J. D. (2001). "Transplantation of clonal neural precursor cells derived from adult human brain establishes functional peripheral myelin in the rat spinal cord." Exp Neurol **167**(1): 27-39.

2 Altman, J. (1969). "Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb." J Comp Neurol **137**(4): 433-57.

3 Altman, J. and Das, G. D. (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." J Comp Neurol **124**(3): 319-35.

4 Altman, J. and Das, G. D. (1965). "Post-natal origin of microneurones in the rat brain." Nature **207**(5000): 953-6.

5 Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., Itskovitz-Eldor, J. and Thomson, J. A. (2000). "Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture." Dev Biol **227**(2): 271-8.

6 Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J. (2006). "Identification of intrinsic determinants of midbrain dopamine neurons." Cell **124**(2): 393-405.

7 Andersson, E. K., Irvin, D. K., Ahlsio, J. and Parmar, M. (2007). "Ngn2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells." Exp Cell Res **313**(6): 1172-80.

8 Arsenijevic, Y., Villemure, J. G., Brunet, J. F., Bloch, J. J., Deglon, N., Kostic, C., Zurn, A. and Aebischer, P. (2001). "Isolation of multipotent neural precursors residing in the cortex of the adult human brain." Exp Neurol **170**(1): 48-62.

9 Aubert, J., Stavridis, M. P., Tweedie, S., O'Reilly, M., Vierlinger, K., Li, M., Ghazal, P., Pratt, T., Mason, J. O., Roy, D. and Smith, A. (2003). "Screening for mammalian neural genes via fluorescence-activated cell sorter purification of neural precursors from Sox1-gfp knock-in mice." Proc Natl Acad Sci U S A **100 Suppl 1**: 11836-41.

10 Ayuso-Sacido, A., Graham, C., Greenfield, J. P. and Boockvar, J. A. (2006). "The duality of epidermal growth factor receptor (EGFR) signaling and neural stem cell phenotype: cell enhancer or cell transformer?" Curr Stem Cell Res Ther **1**(3): 387-94.

11 Azizi, S. A., Stokes, D., Augelli, B. J., DiGirolamo, C. and Prockop, D. J. (1998). "Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats--similarities to astrocyte grafts." Proc Natl Acad Sci U S A **95**(7): 3908-13.

12 Baizabal, J. M., Furlan-Magaril, M., Santa-Olalla, J. and Covarrubias, L. (2003). "Neural stem cells in development and regenerative medicine." Arch Med Res **34**(6): 572-88.

13 Bayer, S. A., Wills, K. V., Triarhou, L. C. and Ghetti, B. (1995). "Time of neuron origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse." Exp Brain Res **105**(2): 191-9.

14 Bayer, S. A., Yackel, J. W. and Puri, P. S. (1982). "Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life." Science **216**(4548): 890-2.

15 Bjorklund, A., Dunnett, S. B., Brundin, P., Stoessl, A. J., Freed, C. R., Breeze, R. E., Levivier, M., Peschanski, M., Studer, L. and Barker, R. (2003). "Neural transplantation for the treatment of Parkinson's disease." Lancet Neurol **2**(7): 437-45.

16 Bjorklund, L. M., Sanchez-Pernaute, R., Chung, S., Andersson, T., Chen, I. Y., McNaught, K. S., Brownell, A. L., Jenkins, B. G., Wahlestedt, C., Kim, K. S. and Isacson, O. (2002). "Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model." Proc Natl Acad Sci U S A **99**(4): 2344-9.

17 Brook, F. A. and Gardner, R. L. (1997). "The origin and efficient derivation of embryonic stem cells in the mouse." Proc Natl Acad Sci U S A **94**(11): 5709-12.

18 Brustle, O., Maskos, U. and McKay, R. D. (1995). "Host-guided migration allows targeted introduction of neurons into the embryonic brain." Neuron **15**(6): 1275-85.

19 Brustle, O., Spiro, A. C., Karram, K., Choudhary, K., Okabe, S. and McKay, R. D. (1997). "In vitro-generated neural precursors participate in mammalian brain development." Proc Natl Acad Sci U S A **94**(26): 14809-14.

20 Burbach, J. P. and Smidt, M. P. (2006). "Molecular programming of stem cells into mesodiencephalic dopaminergic neurons." Trends Neurosci **29**(11): 601-3.

21 Burrows, R. C., Wancio, D., Levitt, P. and Lillien, L. (1997). "Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex." Neuron **19**(2): 251-67.

22 Cai, J., Limke, T. L., Ginis, I. and Rao, M. S. (2003). "Identifying and tracking neural stem cells." Blood Cells Mol Dis **31**(1): 18-27.

23 Cameron, H. A., Woolley, C. S., McEwen, B. S. and Gould, E. (1993). "Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat." Neuroscience **56**(2): 337-44.

24 Campbell, K., Olsson, M. and Bjorklund, A. (1995). "Regional incorporation and site-specific differentiation of striatal precursors transplanted to the embryonic forebrain ventricle." Neuron **15**(6): 1259-73.

25 Carpenter, M. K., Cui, X., Hu, Z. Y., Jackson, J., Sherman, S., Seiger, A. and Wahlberg, L. U. (1999). "In vitro expansion of a multipotent population of human neural progenitor cells." Exp Neurol **158**(2): 265-78.

26 Carvey, P. M., Ling, Z. D., Sortwell, C. E., Pitzer, M. R., McGuire, S. O., Storch, A. and Collier, T. J. (2001). "A clonal line of mesencephalic progenitor cells converted to dopamine neurons by hematopoietic cytokines: a source of cells for transplantation in Parkinson's disease." Exp Neurol **171**(1): 98-108.

27 Castelo-Branco, G., Sousa, K. M., Bryja, V., Pinto, L., Wagner, J. and Arenas, E. (2006). "Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion." Mol Cell Neurosci **31**(2): 251-62.

28 Castelo-Branco, G., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J. and Arenas, E. (2003). "Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a." Proc Natl Acad Sci U S A **100**(22): 12747-52.

29 Cattaneo, E. and McKay, R. (1990). "Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor." Nature **347**(6295): 762-5.

30 Chen, K., Hughes, S. M. and Connor, B. (2007). "Neural progenitor cells derived from the adult rat subventricular zone: characterization and transplantation." Cell Transplant **16**(8): 799-810.

31 Chung, S., Shin, B. S., Hwang, M., Lardaro, T., Kang, U. J., Isacson, O. and Kim, K. S. (2006). "Neural precursors derived from embryonic stem cells, but not those from fetal ventral mesencephalon, maintain the potential to differentiate into dopaminergic neurons after expansion in vitro." Stem Cells **24**(6): 1583-93.

32 Dahlstrand, J., Lardelli, M. and Lendahl, U. (1995). "Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system." Brain Res Dev Brain Res **84**(1): 109-29.

33 Deacon, T., Dinsmore, J., Costantini, L. C., Ratliff, J. and Isacson, O. (1998). "Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation." Exp Neurol **149**(1): 28-41.

34 Dumstrei, K., Nassif, C., Abboud, G., Aryai, A., Aryai, A. and Hartenstein, V. (1998). "EGFR signaling is required for the differentiation and maintenance of neural progenitors along the dorsal midline of the Drosophila embryonic head." Development **125**(17): 3417-26.

35 Dunnett, S. B. and Bjorklund, A. (1999). "Prospects for new restorative and neuroprotective treatments in Parkinson's disease." Nature **399**(6738 Suppl): A32-9.

36 Ellis, P., Fagan, B. M., Magness, S. T., Hutton, S., Taranova, O., Hayashi, S., McMahon, A., Rao, M. and Pevny, L. (2004). "SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult." Dev Neurosci **26**(2-4): 148-65.

37 Eminli, S., Utikal, J., Arnold, K., Jaenisch, R. and Hochedlinger, K. (2008). "Reprogramming of neural progenitor cells into induced pluripotent stem cells in the absence of exogenous Sox2 expression." Stem Cells **26**(10): 2467-74.

38 Ende, N., Weinstein, F., Chen, R. and Ende, M. (2000). "Human umbilical cord blood effect on sod mice (amyotrophic lateral sclerosis)." Life Sci **67**(1): 53-9.

39 Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998). "Neurogenesis in the adult human hippocampus." Nat Med **4**(11): 1313-7.

40 Farkas, L. M., Dunker, N., Roussa, E., Unsicker, K. and Krieglstein, K. (2003). "Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo." J Neurosci **23**(12): 5178-86.

41 Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F. (1998). "Muscle regeneration by bone marrow-derived myogenic progenitors." Science **279**(5356): 1528-30.

42 Ferri, A. L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P. P., Sala, M., DeBiasi, S. and Nicolis, S. K. (2004). "Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain." Development **131**(15): 3805-19.

43 Fishell, G. (1995). "Striatal precursors adopt cortical identities in response to local cues." Development **121**(3): 803-12.

44 Freed, C. R., Breeze, R. E., Rosenberg, N. L., Schneck, S. A., Wells, T. H., Barrett, J. N., Grafton, S. T., Mazziotta, J. C., Eidelberg, D. and Rottenberg, D. A. (1990). "Therapeutic effects of human fetal dopamine cells

transplanted in a patient with Parkinson's disease." Prog Brain Res **82**: 715-21.

45 Fricker, R. A., Carpenter, M. K., Winkler, C., Greco, C., Gates, M. A. and Bjorklund, A. (1999). "Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain." J Neurosci **19**(14): 5990-6005.

46 Friedenstein, A. J., Gorskaja, J. F. and Kulagina, N. N. (1976). "Fibroblast precursors in normal and irradiated mouse hematopoietic organs." Exp Hematol **4**(5): 267-74.

47 Gage, F. H. (2000). "Mammalian neural stem cells." Science **287**(5457): 1433-8.

48 Gage, F. H., Ray, J. and Fisher, L. J. (1995). "Isolation, characterization, and use of stem cells from the CNS." Annu Rev Neurosci **18**: 159-92.

49 Garbuzova-Davis, S., Willing, A. E., Zigova, T., Saporta, S., Justen, E. B., Lane, J. C., Hudson, J. E., Chen, N., Davis, C. D. and Sanberg, P. R. (2003). "Intravenous administration of human umbilical cord blood cells in a mouse model of amyotrophic lateral sclerosis: distribution, migration, and differentiation." J Hematother Stem Cell Res **12**(3): 255-70.

50 Gensburger, C., Labourdette, G. and Sensenbrenner, M. (1987). "Brain basic fibroblast growth factor stimulates the proliferation of rat neuronal precursor cells in vitro." FEBS Lett **217**(1): 1-5.

51 Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U. and Stein, H. (1984). "Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67." J Immunol **133**(4): 1710-5.

52 Gotz, M. and Huttner, W. B. (2005). "The cell biology of neurogenesis." Nat Rev Mol Cell Biol **6**(10): 777-88.

53 Gould, E., Reeves, A. J., Graziano, M. S. and Gross, C. G. (1999). "Neurogenesis in the neocortex of adult primates." Science **286**(5439): 548-52.

54 Graves, B. J., Hatada, M. H., Hendrickson, W. A., Miller, J. K., Madison, V. S. and Satow, Y. (1990). "Structure of interleukin 1 alpha at 2.7-A resolution." Biochemistry **29**(11): 2679-84.

55 Gritti, A., Bonfanti, L., Doetsch, F., Caille, I., Alvarez-Buylla, A., Lim, D. A., Galli, R., Verdugo, J. M., Herrera, D. G. and Vescovi, A. L. (2002). "Multipotent neural stem cells reside into the rostral extension and olfactory bulb of adult rodents." J Neurosci **22**(2): 437-45.

56 Gritti, A., Parati, E. A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D. J., Roisen, F., Nickel, D. D. and Vescovi, A. L.

(1996). "Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor." J Neurosci **16**(3): 1091-100.

57 Haas, S., Weidner, N. and Winkler, J. (2005). "Adult stem cell therapy in stroke." Curr Opin Neurol **18**(1): 59-64.

58 Habisch, H. J., Janowski, M., Binder, D., Kuzma-Kozakiewicz, M., Widmann, A., Habich, A., Schwalenstocker, B., Hermann, A., Brenner, R., Lukomska, B., Domanska-Janik, K., Ludolph, A. C. and Storch, A. (2007). "Intrathecal application of neuroectodermally converted stem cells into a mouse model of ALS: limited intraparenchymal migration and survival narrows therapeutic effects." J Neural Transm **114**(11): 1395-406.

59 Hartfuss, E., Galli, R., Heins, N. and Gotz, M. (2001). "Characterization of CNS precursor subtypes and radial glia." Dev Biol **229**(1): 15-30.

60 Haubensak, W., Attardo, A., Denk, W. and Huttner, W. B. (2004). "Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis." Proc Natl Acad Sci U S A **101**(9): 3196-201.

61 Hermann, A., Gastl, R., Liebau, S., Popa, M. O., Fiedler, J., Boehm, B. O., Maisel, M., Lerche, H., Schwarz, J., Brenner, R. and Storch, A. (2004). "Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells." J Cell Sci **117**(Pt 19): 4411-22.

62 Hermann, A., Gerlach, M., Schwarz, J. and Storch, A. (2004). "Neurorestoration in Parkinson's disease by cell replacement and endogenous regeneration." Expert Opin Biol Ther **4**(2): 131-43.

63 Hermann, A., Maisel, M., Liebau, S., Gerlach, M., Kleger, A., Schwarz, J., Kim, K. S., Antoniadis, G., Lerche, H. and Storch, A. (2006). "Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells." J Neurochem **98**(2): 629-40.

64 Hermann, A., Maisel, M. and Storch, A. (2006). "Epigenetic conversion of human adult bone mesodermal stromal cells into neuroectodermal cell types for replacement therapy of neurodegenerative disorders." Expert Opin Biol Ther **6**(7): 653-70.

65 Hermann, A., Maisel, M., Wegner, F., Liebau, S., Kim, D. W., Gerlach, M., Schwarz, J., Kim, K. S. and Storch, A. (2006). "Multipotent neural stem cells from the adult tegmentum with dopaminergic potential develop essential properties of functional neurons." Stem Cells **24**(4): 949-64.

66 Hitoshi, S., Tropepe, V., Ekker, M. and van der Kooy, D. (2002). "Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain." Development **129**(1): 233-44.

67 Hodges, H., Veizovic, T., Bray, N., French, S. J., Rashid, T. P., Chadwick, A., Patel, S. and Gray, J. A. (2000). "Conditionally immortal neuroepithelial stem cell grafts reverse age-associated memory impairments in rats." Neuroscience **101**(4): 945-55.

68 Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W. and Melton, D. A. (2008). "Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2." Nat Biotechnol **26**(11): 1269-75.

69 Hynes, M. and Rosenthal, A. (2000). "Embryonic stem cells go dopaminergic." Neuron **28**(1): 11-4.

70 Isacson, O., Bjorklund, L. M. and Schumacher, J. M. (2003). "Toward full restoration of synaptic and terminal function of the dopaminergic system in Parkinson's disease by stem cells." Ann Neurol **53 Suppl 3**: S135-46; discussion S46-8.

71 Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A. and Verfaillie, C. M. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature **418**(6893): 41-9.

72 Johansson, C. B., Svensson, M., Wallstedt, L., Janson, A. M. and Frisen, J. (1999). "Neural stem cells in the adult human brain." Exp Cell Res **253**(2): 733-6.

73 Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M. and McKay, R. D. (1996). "Single factors direct the differentiation of stem cells from the fetal and adult central nervous system." Genes Dev **10**(24): 3129-40.

74 Kania, G., Corbeil, D., Fuchs, J., Tarasov, K. V., Blyszczuk, P., Huttner, W. B., Boheler, K. R. and Wobus, A. M. (2005). "Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors." Stem Cells **23**(6): 791-804.

75 Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Nishikawa, S. I. and Sasai, Y. (2000). "Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity." Neuron **28**(1): 31-40.

76 Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., Haruta, M., Takahashi, M., Yoshikawa, K., Nishikawa, S., Nakatsuji, N. and Sasai, Y. (2002). "Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity." Proc Natl Acad Sci U S A **99**(3): 1580-5.

77 Key, G., Kubbutat, M. H. and Gerdes, J. (1994). "Assessment of cell proliferation by means of an enzyme-linked immunosorbent assay based on the detection of the Ki-67 protein." J Immunol Methods **177**(1-2): 113-7.

78 Kilpatrick, T. J. and Bartlett, P. F. (1993). "Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation." Neuron **10**(2): 255-65.

79 Kim, H. T., Kim, I. S., Lee, I. S., Lee, J. P., Snyder, E. Y. and Park, K. I. (2006). "Human neurospheres derived from the fetal central nervous system are regionally and temporally specified but are not committed." Exp Neurol **199**(1): 222-35.

80 Kim, J. B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M. J., Ruau, D., Han, D. W., Zenke, M. and Scholer, H. R. (2008). "Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors." Nature **454**(7204): 646-50.

81 Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., Son, H., Lee, Y. S., Studer, L., McKay, R. and Lee, S. H. (2003). "Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression." J Neurochem **85**(6): 1443-54.

82 Kim, T. E., Lee, H. S., Lee, Y. B., Hong, S. H., Lee, Y. S., Ichinose, H., Kim, S. U. and Lee, M. A. (2003). "Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell." Biochem Biophys Res Commun **305**(4): 1040-8.

83 Ko, J. Y., Lee, J. Y., Park, C. H. and Lee, S. H. (2005). "Effect of cell-density on in-vitro dopaminergic differentiation of mesencephalic precursor cells." Neuroreport **16**(5): 499-503.

84 Ko, J. Y., Park, C. H., Koh, H. C., Cho, Y. H., Kyhm, J. H., Kim, Y. S., Lee, I., Lee, Y. S. and Lee, S. H. (2007). "Human embryonic stem cell-derived neural precursors as a continuous, stable, and on-demand source for human dopamine neurons." J Neurochem **103**(4): 1417-29.

85 Komitova, M. and Eriksson, P. S. (2004). "Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain." Neurosci Lett **369**(1): 24-7.

86 Kordower, J. H., Freeman, T. B., Snow, B. J., Vingerhoets, F. J., Mufson, E. J., Sanberg, P. R., Hauser, R. A., Smith, D. A., Nauert, G. M., Perl, D. P. and et al. (1995). "Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease." N Engl J Med **332**(17): 1118-24.

87 Kornblum, H. I., Hussain, R. J., Bronstein, J. M., Gall, C. M., Lee, D. C. and Seroogy, K. B. (1997). "Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain." J Comp Neurol **380**(2): 243-61.

88 Kukekov, V. G., Laywell, E. D., Suslov, O., Davies, K., Scheffler, B., Thomas, L. B., O'Brien, T. F., Kusakabe, M. and Steindler, D. A. (1999). "Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain." Exp Neurol **156**(2): 333-44.

89 Kukekov, V. G., Laywell, E. D., Thomas, L. B. and Steindler, D. A. (1997). "A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia." Glia **21**(4): 399-407.

90 Lee, A., Kessler, J. D., Read, T. A., Kaiser, C., Corbeil, D., Huttner, W. B., Johnson, J. E. and Wechsler-Reya, R. J. (2005). "Isolation of neural stem cells from the postnatal cerebellum." Nat Neurosci **8**(6): 723-9.

91 Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000). "Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells." Nat Biotechnol **18**(6): 675-9.

92 Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990). "CNS stem cells express a new class of intermediate filament protein." Cell **60**(4): 585-95.

93 Levison, S. W. and Goldman, J. E. (1993). "Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain." Neuron **10**(2): 201-12.

94 Levkovits, I. a. W., H. (1999). Limiting Dilution Analysis of Cells of the Immune System. Oxford.

95 Lie, D. C., Dziewczapolski, G., Willhoite, A. R., Kaspar, B. K., Shults, C. W. and Gage, F. H. (2002). "The adult substantia nigra contains progenitor cells with neurogenic potential." J Neurosci **22**(15): 6639-49.

96 Lillien, L. and Raphael, H. (2000). "BMP and FGF regulate the development of EGF-responsive neural progenitor cells." Development **127**(22): 4993-5005.

97 Lin, J. H., Takano, T., Arcuino, G., Wang, X., Hu, F., Darzynkiewicz, Z., Nunes, M., Goldman, S. A. and Nedergaard, M. (2007). "Purinergic signaling regulates neural progenitor cell expansion and neurogenesis." Dev Biol **302**(1): 356-66.

98 Lindvall, O. (1997). "Neural transplantation: a hope for patients with Parkinson's disease." Neuroreport **8**(14): iii-x.

99 Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K. L., Sawle, G., Rothwell, J. C., Marsden, C. D. and et al. (1990). "Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease." Science **247**(4942): 574-7.

100 Lindvall, O., Kokaia, Z. and Martinez-Serrano, A. (2004). "Stem cell therapy for human neurodegenerative disorders-how to make it work." Nat Med **10 Suppl**: S42-50.

101 Ling, Z. D., Potter, E. D., Lipton, J. W. and Carvey, P. M. (1998). "Differentiation of mesencephalic progenitor cells into dopaminergic neurons by cytokines." Exp Neurol **149**(2): 411-23.

102 Livak, K. J. and Schmittgen, T. D. (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-8.

103 Lois, C. and Alvarez-Buylla, A. (1993). "Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia." Proc Natl Acad Sci U S A **90**(5): 2074-7.

104 Lois, C. and Alvarez-Buylla, A. (1994). "Long-distance neuronal migration in the adult mammalian brain." Science **264**(5162): 1145-8.

105 Loo, D. T., Fuquay, J. I., Rawson, C. L. and Barnes, D. W. (1987). "Extended culture of mouse embryo cells without senescence: inhibition by serum." Science **236**(4798): 200-2.

106 Luskin, M. B., Parnavelas, J. G. and Barfield, J. A. (1993). "Neurons, astrocytes, and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: an ultrastructural analysis of clonally related cells." J Neurosci **13**(4): 1730-50.

107 Maciaczyk, J., Singec, I., Maciaczyk, D. and Nikkhah, G. (2008). "Combined use of BDNF, ascorbic acid, low oxygen, and prolonged differentiation time generates tyrosine hydroxylase-expressing neurons after long-term in vitro expansion of human fetal midbrain precursor cells." Exp Neurol **213**(2): 354-62.

108 Maisel, M., Herr, A., Milosevic, J., Hermann, A., Habisch, H. J., Schwarz, S., Kirsch, M., Antoniadis, G., Brenner, R., Hallmeyer-Elgner, S., Lerche, H., Schwarz, J. and Storch, A. (2007). "Transcription profiling of adult and fetal human neuroprogenitors identifies divergent paths to maintain the neuroprogenitor cell state." Stem Cells.

109 Makino, S., Fukuda, K., Miyoshi, S., Konishi, F., Kodama, H., Pan, J., Sano, M., Takahashi, T., Hori, S., Abe, H., Hata, J., Umezawa, A. and Ogawa, S. (1999). "Cardiomyocytes can be generated from marrow stromal cells in vitro." J Clin Invest **103**(5): 697-705.

110 Malatesta, P., Hartfuss, E. and Gotz, M. (2000). "Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage." Development **127**(24): 5253-63.

111 Maric, D., Maric, I., Chang, Y. H. and Barker, J. L. (2003). "Prospective cell sorting of embryonic rat neural stem cells and neuronal and glial

progenitors reveals selective effects of basic fibroblast growth factor and epidermal growth factor on self-renewal and differentiation." J Neurosci **23**(1): 240-51.

112 Martinez-Cerdeno, V., Noctor, S. C. and Kriegstein, A. R. (2006). "The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex." Cereb Cortex **16 Suppl 1**: i152-61.

113 Martinez-Serrano, A. and Bjorklund, A. (1997). "Immortalized neural progenitor cells for CNS gene transfer and repair." Trends Neurosci **20**(11): 530-8.

114 Matsushita, N., Okada, H., Yasoshima, Y., Takahashi, K., Kiuchi, K. and Kobayashi, K. (2002). "Dynamics of tyrosine hydroxylase promoter activity during midbrain dopaminergic neuron development." J Neurochem **82**(2): 295-304.

115 Mayer-Proschel, M., Kalyani, A. J., Mujtaba, T. and Rao, M. S. (1997). "Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells." Neuron **19**(4): 773-85.

116 McKay, R. (1997). "Stem cells in the central nervous system." Science **276**(5309): 66-71.

117 Milosevic, J., Brandt, A., Roemuss, U., Arnold, A., Wegner, F., Schwarz, S. C., Storch, A., Zimmermann, H. and Schwarz, J. (2006). "Uracil nucleotides stimulate human neural precursor cell proliferation and dopaminergic differentiation: involvement of MEK/ERK signalling." J Neurochem **99**(3): 913-23.

118 Moe, M. C., Westerlund, U., Varghese, M., Berg-Johnsen, J., Svensson, M. and Langmoen, I. A. (2005). "Development of neuronal networks from single stem cells harvested from the adult human brain." Neurosurgery **56**(6): 1182-8; discussion 88-90.

119 Morshead, C. M. and van der Kooy, D. (1992). "Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain." J Neurosci **12**(1): 249-56.

120 Muraoka, K., Shingo, T., Yasuhara, T., Kameda, M., Yuen, W. J., Uozumi, T., Matsui, T., Miyoshi, Y. and Date, I. (2008). "Comparison of the therapeutic potential of adult and embryonic neural precursor cells in a rat model of Parkinson disease." J Neurosurg **108**(1): 149-59.

121 Murzin, A. G., Lesk, A. M. and Chothia, C. (1992). "beta-Trefoil fold. Patterns of structure and sequence in the Kunitz inhibitors interleukins-1 beta and 1 alpha and fibroblast growth factors." J Mol Biol **223**(2): 531-43.

122 Nishino, H., Hida, H., Takei, N., Kumazaki, M., Nakajima, K. and Baba, H. (2000). "Mesencephalic neural stem (progenitor) cells develop to

dopaminergic neurons more strongly in dopamine-depleted striatum than in intact striatum." Exp Neurol **164**(1): 209-14.

123 Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K. and Kriegstein, A. R. (2002). "Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia." J Neurosci **22**(8): 3161-73.

124 Nunes, M. C., Roy, N. S., Keyoung, H. M., Goodman, R. R., McKhann, G., 2nd, Jiang, L., Kang, J., Nedergaard, M. and Goldman, S. A. (2003). "Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain." Nat Med **9**(4): 439-47.

125 Olanow, C. W., Kordower, J. H. and Freeman, T. B. (1996). "Fetal nigral transplantation as a therapy for Parkinson's disease." Trends Neurosci **19**(3): 102-9.

126 Orlic, D., Kajstura, J., Chimenti, S., Bodine, D. M., Leri, A. and Anversa, P. (2001). "Transplanted adult bone marrow cells repair myocardial infarcts in mice." Ann N Y Acad Sci **938**: 221-9; discussion 29-30.

127 Ortega-Perez, I., Murray, K. and Lledo, P. M. (2007). "The how and why of adult neurogenesis." J Mol Histol **38**(6): 555-62.

128 Ostenfeld, T., Joly, E., Tai, Y. T., Peters, A., Caldwell, M., Jauniaux, E. and Svendsen, C. N. (2002). "Regional specification of rodent and human neurospheres." Brain Res Dev Brain Res **134**(1-2): 43-55.

129 Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F. and Gage, F. H. (1999). "Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS." J Neurosci **19**(19): 8487-97.

130 Palmer, T. D., Ray, J. and Gage, F. H. (1995). "FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain." Mol Cell Neurosci **6**(5): 474-86.

131 Palmer, T. D., Takahashi, J. and Gage, F. H. (1997). "The adult rat hippocampus contains primordial neural stem cells." Mol Cell Neurosci **8**(6): 389-404.

132 Parish, C. L., Castelo-Branco, G., Rawal, N., Tonnesen, J., Sorensen, A. T., Salto, C., Kokaia, M., Lindvall, O. and Arenas, E. (2008). "Wnt5a-treated midbrain neural stem cells improve dopamine cell replacement therapy in parkinsonian mice." J Clin Invest **118**(1): 149-60.

133 Perrone-Capano, C. and Di Porzio, U. (2000). "Genetic and epigenetic control of midbrain dopaminergic neuron development." Int J Dev Biol **44**(6): 679-87.

134 Pfenninger, C. V., Roschupkina, T., Hertwig, F., Kottwitz, D., Englund, E., Bengzon, J., Jacobsen, S. E. and Nuber, U. A. (2007). "CD133 is not present on neurogenic astrocytes in the adult subventricular zone, but on embryonic neural stem cells, ependymal cells, and glioblastoma cells." Cancer Res **67**(12): 5727-36.

135 Pluchino, S., Furlan, R. and Martino, G. (2004). "Cell-based remyelinating therapies in multiple sclerosis: evidence from experimental studies." Curr Opin Neurol **17**(3): 247-55.

136 Potter, E. D., Ling, Z. D. and Carvey, P. M. (1999). "Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons." Cell Tissue Res **296**(2): 235-46.

137 Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M., Martinez, S., Arenas, E., Simeone, A. and Wurst, W. (2006). "A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo." Development **133**(1): 89-98.

138 Priestle, J. P., Schar, H. P. and Grutter, M. G. (1989). "Crystallographic refinement of interleukin 1 beta at 2.0 A resolution." Proc Natl Acad Sci U S A **86**(24): 9667-71.

139 Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L. and Verfaillie, C. M. (2001). "Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells." Blood **98**(9): 2615-25.

140 Reynolds, B. A., Tetzlaff, W. and Weiss, S. (1992). "A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes." J Neurosci **12**(11): 4565-74.

141 Reynolds, B. A. and Weiss, S. (1992). "Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system." Science **255**(5052): 1707-10.

142 Reynolds, B. A. and Weiss, S. (1996). "Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell." Dev Biol **175**(1): 1-13.

143 Richardson, R. M., Broaddus, W. C., Holloway, K. L. and Fillmore, H. L. (2005). "Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline." Brain Res **1032**(1-2): 11-22.

144 Rietze, R. L., Valcanis, H., Brooker, G. F., Thomas, T., Voss, A. K. and Bartlett, P. F. (2001). "Purification of a pluripotent neural stem cell from the adult mouse brain." Nature **412**(6848): 736-9.

145 Roussa, E., Wiehle, M., Dunker, N., Becker-Katins, S., Oehlke, O. and Krieglstein, K. (2006). "Transforming growth factor beta is required for differentiation of mouse mesencephalic progenitors into dopaminergic

neurons in vitro and in vivo: ectopic induction in dorsal mesencephalon." Stem Cells **24**(9): 2120-9.

146 Roy, N. S., Wang, S., Jiang, L., Kang, J., Benraiss, A., Harrison-Restelli, C., Fraser, R. A., Couldwell, W. T., Kawaguchi, A., Okano, H., Nedergaard, M. and Goldman, S. A. (2000). "In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus." Nat Med **6**(3): 271-7.

147 Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D. and Gage, F. H. (1999). "Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain." Development **126**(18): 4017-26.

148 Sanchez-Ramos, J. R. (2002). "Neural cells derived from adult bone marrow and umbilical cord blood." J Neurosci Res **69**(6): 880-93.

149 Sanchez-Ramos, J. R., Song, S., Kamath, S. G., Zigova, T., Willing, A., Cardozo-Pelaez, F., Stedeford, T., Chopp, M. and Sanberg, P. R. (2001). "Expression of neural markers in human umbilical cord blood." Exp Neurol **171**(1): 109-15.

150 Sawamoto, K., Nakao, N., Kakishita, K., Ogawa, Y., Toyama, Y., Yamamoto, A., Yamaguchi, M., Mori, K., Goldman, S. A., Itakura, T. and Okano, H. (2001). "Generation of dopaminergic neurons in the adult brain from mesencephalic precursor cells labeled with a nestin-GFP transgene." J Neurosci **21**(11): 3895-903.

151 Schofield, R. (1978). "The relationship between the spleen colony-forming cell and the haemopoietic stem cell." Blood Cells **4**(1-2): 7-25.

152 Seaberg, R. M. and van der Kooy, D. (2002). "Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors." J Neurosci **22**(5): 1784-93.

153 Sekiya, I., Larson, B. L., Smith, J. R., Pochampally, R., Cui, J. G. and Prockop, D. J. (2002). "Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality." Stem Cells **20**(6): 530-41.

154 Selander, L. and Edlund, H. (2002). "Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas." Mech Dev **113**(2): 189-92.

155 Shihabuddin, L. S. (2002). "Adult rodent spinal cord derived neural stem cells. Isolation and characterization." Methods Mol Biol **198**: 67-77.

156 Shingo, T., Sorokan, S. T., Shimazaki, T. and Weiss, S. (2001). "Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells." J Neurosci **21**(24): 9733-43.

157 Smidt, M. P., Asbreuk, C. H., Cox, J. J., Chen, H., Johnson, R. L. and Burbach, J. P. (2000). "A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b." Nat Neurosci **3**(4): 337-41.

158 Smith, R., Bagga, V. and Fricker-Gates, R. A. (2003). "Embryonic neural progenitor cells: the effects of species, region, and culture conditions on long-term proliferation and neuronal differentiation." J Hematother Stem Cell Res **12**(6): 713-25.

159 Storch, A., Lester, H. A., Boehm, B. O. and Schwarz, J. (2003). "Functional characterization of dopaminergic neurons derived from rodent mesencephalic progenitor cells." J Chem Neuroanat **26**(2): 133-42.

160 Storch, A., Paul, G., Csete, M., Boehm, B. O., Carvey, P. M., Kupsch, A. and Schwarz, J. (2001). "Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells." Exp Neurol **170**(2): 317-25.

161 Storch, A., Sabolek, M., Milosevic, J., Schwarz, S. C. and Schwarz, J. (2004). "Midbrain-derived neural stem cells: from basic science to therapeutic approaches." Cell Tissue Res **318**(1): 15-22.

162 Storch, A. and Schwarz, J. (2002). "Neural stem cells and Parkinson's disease." J Neurol **249 Suppl 3**: III/30-2.

163 Studer, L., Csete, M., Lee, S. H., Kabbani, N., Walikonis, J., Wold, B. and McKay, R. (2000). "Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen." J Neurosci **20**(19): 7377-83.

164 Studer, L., Tabar, V. and McKay, R. D. (1998). "Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats." Nat Neurosci **1**(4): 290-5.

165 Stull, N. D. and Iacovitti, L. (2001). "Sonic hedgehog and FGF8: inadequate signals for the differentiation of a dopamine phenotype in mouse and human neurons in culture." Exp Neurol **169**(1): 36-43.

166 Sun, Z. H., Lai, Y. L., Zeng, W. W., Zhao, D., Ye, Z. W., Zuo, H. C. and Xie, Z. P. (2003). "Mesencephalic progenitors can improve rotational behavior and reconstruct nigrostriatal pathway in PD rats." Acta Neurochir Suppl **87**: 175-80.

167 Sun, Z. H., Lai, Y. L., Zeng, W. W., Zhao, D., Zuo, H. C. and Xie, Z. P. (2003). "Neural stem/progenitor cells survive and differentiate better in PD rats than in normal rats." Acta Neurochir Suppl **87**: 169-74.

168 Svendsen, C. N., Caldwell, M. A. and Ostenfeld, T. (1999). "Human neural stem cells: isolation, expansion and transplantation." Brain Pathol **9**(3): 499-513.

169 Svendsen, C. N., Caldwell, M. A., Shen, J., ter Borg, M. G., Rosser, A. E., Tyers, P., Karmiol, S. and Dunnett, S. B. (1997). "Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease." Exp Neurol **148**(1): 135-46.

170 Svendsen, C. N., Clarke, D. J., Rosser, A. E. and Dunnett, S. B. (1996). "Survival and differentiation of rat and human epidermal growth factor-responsive precursor cells following grafting into the lesioned adult central nervous system." Exp Neurol **137**(2): 376-88.

171 Svendsen, C. N. and Langston, J. W. (2004). "Stem cells for Parkinson disease and ALS: replacement or protection?" Nat Med **10**(3): 224-5.

172 Svendsen, C. N., Skepper, J., Rosser, A. E., ter Borg, M. G., Tyres, P. and Ryken, T. (1997). "Restricted growth potential of rat neural precursors as compared to mouse." Brain Res Dev Brain Res **99**(2): 253-8.

173 Svendsen, C. N. and Smith, A. G. (1999). "New prospects for human stem-cell therapy in the nervous system." Trends Neurosci **22**(8): 357-64.

174 Taupin, P. (2006). "Neural progenitor and stem cells in the adult central nervous system." Ann Acad Med Singapore **35**(11): 814-20.

175 Taupin, P. and Gage, F. H. (2002). "Adult neurogenesis and neural stem cells of the central nervous system in mammals." J Neurosci Res **69**(6): 745-9.

176 Toma, J. G., Akhavan, M., Fernandes, K. J., Barnabe-Heider, F., Sadikot, A., Kaplan, D. R. and Miller, F. D. (2001). "Isolation of multipotent adult stem cells from the dermis of mammalian skin." Nat Cell Biol **3**(9): 778-84.

177 Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H. and Weissman, I. L. (2000). "Direct isolation of human central nervous system stem cells." Proc Natl Acad Sci U S A **97**(26): 14720-5.

178 Vescovi, A. L., Parati, E. A., Gritti, A., Poulin, P., Ferrario, M., Wanke, E., Frolichsthal-Schoeller, P., Cova, L., Arcellana-Panlilio, M., Colombo, A. and Galli, R. (1999). "Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation." Exp Neurol **156**(1): 71-83.

179 Vicario-Abejon, C., Cunningham, M. G. and McKay, R. D. (1995). "Cerebellar precursors transplanted to the neonatal dentate gyrus express features characteristic of hippocampal neurons." J Neurosci **15**(10): 6351-63.

180 Vigers, G. P., Caffes, P., Evans, R. J., Thompson, R. C., Eisenberg, S. P. and Brandhuber, B. J. (1994). "X-ray structure of interleukin-1 receptor antagonist at 2.0-A resolution." J Biol Chem **269**(17): 12874-9.

181 Vitkovic, L., Bockaert, J. and Jacque, C. (2000). ""Inflammatory" cytokines: neuromodulators in normal brain?" J Neurochem **74**(2): 457-71.

182 Vogel, W., Grunebach, F., Messam, C. A., Kanz, L., Brugger, W. and Buhring, H. J. (2003). "Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells." Haematologica **88**(2): 126-33.

183 Wagner, J., Akerud, P., Castro, D. S., Holm, P. C., Canals, J. M., Snyder, E. Y., Perlmann, T. and Arenas, E. (1999). "Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes." Nat Biotechnol **17**(7): 653-9.

184 Westerlund, U., Moe, M. C., Varghese, M., Berg-Johnsen, J., Ohlsson, M., Langmoen, I. A. and Svensson, M. (2003). "Stem cells from the adult human brain develop into functional neurons in culture." Exp Cell Res **289**(2): 378-83.

185 Whittemore, S. R., Morassutti, D. J., Walters, W. M., Liu, R. H. and Magnuson, D. S. (1999). "Mitogen and substrate differentially affect the lineage restriction of adult rat subventricular zone neural precursor cell populations." Exp Cell Res **252**(1): 75-95.

186 Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. and Rosenthal, A. (1998). "FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate." Cell **93**(5): 755-66.

187 Zietlow, R., Lane, E. L., Dunnett, S. B. and Rosser, A. E. (2008). "Human stem cells for CNS repair." Cell Tissue Res **331**(1): 301-22.