Brain Microenvironment and its Influence on Gene Expression and Differentiation of Murine Embryonic Stem Cells – Implications for Cell Replacement Strategies

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Für meine Eltern

ABSTRACT

The discovery of pluripotent embryonic stem (ES) cells more than 20 years ago marked the beginning of a new era in developmental biology and biomedical research. The outstanding endogenous attributes of embryonic stem cells and the availability of *in vitro* ES cell differentiation protocols emphasise the great potential of these cells for clinical application in CNS disorders. Particularly for the treatment of disease states in which no clinical therapy has proven effective to date, such as traumatic brain injury, stem cell-based cell replacement strategies are advancing. However, in animal models of traumatic brain injury it has been shown that soluble signalling molecules, extracellular matrix-associated activities and the cellular environment at the site of implantation exert great influence on engrafted stem cells and can be decisive for success or failure of functional engraftment.

This doctoral thesis aims at elucidating the role of naïve and pathological environmental conditions on plasticity of murine embryonic stem cells. Utilising a sophisticated *in vitro* approach i) the influence of endothelial and astroglial cells on stem cell self-renewal and differentiation capacity, ii) the impact of traumatic brain injury-associated environment on stem cell fate and iii) the source of motor functional improvements, observed after stem cell transplantation into the traumatically injured brain *in vivo* were investigated. To achieve this, embryonic stem cells were co-cultured with cerebral endothelial or astroglial cells, both belonging to the neural stem cell niche *in vivo*, or treated with tissue extract derived from traumatised or healthy rat brain, respectively. Subsequently, embryonic stem cells were analysed for expression of developmental marker genes (RT-PCR), mitotic activity (FACS), production of neurotrophic factors (ELISA) and morphological appearance (microscopy). Results indicate that environmental conditions, both on a cellular and molecular level, distinctively manipulate stem cell fate. It is demonstrated that neuronal lineage commitment in ES cells is mainly mediated via humoral factors present in conditioned medium, whereas the vicinity of living endothelial cells and astrocytes delays differentiation activities and instead promotes oligodendroglial cell fates. Furthermore, it is shown that traumatic brain injury-related parameters support the rapid differentiation of single stem cells to mature neuronal phenotypes. However, many cells are lost due to the deleterious effects of trauma environment. Moreover, cerebral tissue extract is shown to either enhance or restrict the release of trophic factors by embryonic stem cells, and additionally, has the ability to induce growth factor production in fibroblasts. Thus, co-transplanted fibroblasts are proposed to represent an alternative source for functional recovery observed after ES cell transplantation. With its findings, this study tries to bridge a gap between basic biological research and clinical science in a common effort to aid the human patient finally.

ZUSAMMENFASSUNG

Die erstmalige Isolation pluripotenter embryonaler Stammzellen vor mehr als 20 Jahren kennzeichnete weltweit den Beginn einer neuen Ära in der entwicklungsbiologischen und medizinischen Forschung. Die außergewöhnlichen endogenen Eigenschaften embryonaler Stammzellen, ihre Eignung zu unbegrenzter Vermehrung *in vitro* und ihre Fähigkeit Zellen verschiedensten Gewebeursprungs hervorzubringen, unterstreichen das große Potential dieser Zellen für eine klinische Anwendung. Zellersatz durch Stammzelltransplantation eröffnet vor allem bei ZNS-Verletzungen, wie z.B. bei Schädel-Hirn-Trauma, neue Behandlungsmöglichkeiten. Es konnte jedoch gezeigt werden, dass die Interaktion transplantierter Zellen mit humoralen Faktoren, extrazellulären Matrixmolekülen und dem zellulären Umfeld vor Ort von entscheidender Bedeutung für Erfolg oder Misserfolg einer durchgeführten Transplantationstherapie sein kann.

Ziel dieser Dissertation ist es, den Einfluss des extrazellulären Milieus, unter gesunden und pathologischen Bedingungen, auf die Plastizität muriner embryonaler Stammzellen aufzuklären. Mittels eines *in vitro*-Modells wurde i) der Einfluss der lokalen Umgebung, der so genannten Nische, auf Selbsterhaltung und Differenzierung embryonaler Stammzellen untersucht, ii) die Auswirkungen pathologischer Umwelteinflüsse auf das Zellschicksal embryonaler Stammzellen erforscht und iii) die Ursache funktionaler Verbesserungen, die nach Transplantation von Stammzellen *in vivo* beobachtet wurden, analysiert. Dazu wurden murine embryonale Stammzellen entweder mit zerebralen Endothelzellen oder Astroglia co-kultiviert, oder mit Gewebeextrakt behandelt, der von gesundem bzw. Schädel-Hirn-traumatisiertem Rattenhirn gewonnen wurde. Anschließend wurden die Stammzellen auf die Expression verschiedener entwicklungsspezifischer Gene (RT-PCR), auf ihre Proliferationsfähigkeit und ihre Fähigkeit zur Produktion neurotropher Faktoren (ELISA), sowie auf sichtbare morphologische Veränderungen (Mikroskopie) untersucht. Die Ergebnisse dieser Studie zeigen, dass zelluläre und molekulare Umgebungsbedingungen das Schicksal embryonaler Stammzellen unterschiedlich beeinflussen. Während die Induktion neuronaler Differenzierungsvorgängen vornehmlich über humorale Signale gesteuert wird, die in konditioniertem Medium vorhanden sind, werden Differenzierungsvorgänge durch in unmittelbarer Nähe wachsende Endothelzellen und Astrozyten verlangsamt. Außerdem bevorzugen in Co-Kultur gewachsene Stammzellen ein gliales Zellschicksal. Des Weiteren kann unter Einfluss eines Schädel-Hirn-Traumas, im Gegensatz zu nativen Bedingungen, eine beschleunigte neuronale Differenzierung einzelner Stammzellen beobachtet werden. Jedoch kommt es durch die Einwirkung verletzungsbedingter Parameter auch zu großen Zellverlusten. Veränderte Kulturbedingungen beeinflussen auch die Produktion neurotropher Faktoren. So wurde unter Gewebeextrakteinfluss sowohl eine Steigerung als auch eine Verringerung der Neurothophinsekretionsrate in Stammzellen beobachtet. Weiterhin werden Fibroblasten durch Faktoren im Gewebeextrakt zur Produktion von Wachstumsfaktoren angeregt. Daher muss die Möglichkeit in Betracht gezogen werden, dass co-transplantierte Fibroblasten für die Funktionsverbesserungen, die nach Stammzelltransplantation beobachtet wurden, verantwortlich sind. Die Erkenntnisse dieser Doktorarbeit können somit für die Beurteilung und Verbesserung stammzellbasierter Zellersatztherapien herangezogen werden und vereinen experimentelle biologische Grundlagenforschung und klinische Anwendung im Dienste des Patienten.

ABBREVIATIONS

°C	Degree Centigrade
µg	Microgram
µL	Microliter
µM	Micromole
ATCC	The American Type Culture Collection
atm	Standard atmosphere
bp	Base pair(s)
cDNA	Complementary DNA
DAB	3, 3'-diaminobenzidine
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acids
dNTP	Deoxyribonucleoside triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
E	Embryonic day
ECACC	The European Collection of Cell Cultures
$F(ab')_2$	Fragment antigen-binding
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram
g	Standard acceleration due to gravity (9.81 m/s^2)
GFP	Green fluorescent protein
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
L	Liter
M	Molarity
mg	Milligram
min	Minute(s)
mL	Milliliter
mm	Millimeter
mM	Millimole
ng	Nanogram
nm	Nanometer
PerCP	Peridinin-chlorophyll-protein complex
pH	"potentia hydrogenii", negative logarithm of the hydrogen ion concentration
pmol	Pico mole
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	Second(s)
SEM	Standard error of the mean
Taq	Thermophilius aquaticus
U	Unit(s)
UV	Ultra-violet
V	Volt
w	With
w/o	Without

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1 INTRODUCTION

One of today's biggest challenges in molecular biological and biomedical research is the dissection of early developmental and regenerative processes that are responsible for the functional complexity of the mammalian central nervous system (CNS). The CNS assembly, consisting of a network of distinct cellular populations interwoven to a multifaceted functional entity contributes to our limited understanding. The elucidation of mechanisms and signalling pathways guiding neurogenesis and regeneration are the key to developing effective therapies for the treatment of various human neurodegenerative diseases and injuries (e.g. Morbus Parkinson, Morbus Alzheimer, Spinal Cord and Traumatic Brain Injury). The aetiology of neurodegenerative disease is associated with the progressive loss or damage of neuronal tissue leading to neuronal and behavioural deficits in the patient and is to date generally regarded as incurable. Moreover, the regenerative potential of the CNS is regarded as rather limited, and generally, neuronal populations cannot be replenished following cell death. Although many attempts to inhibit damaging pathological processes or to stimulate pathophysiological pathways e.g. by the administration of neuroprotective agents have been developed, these approaches could only attenuate post-traumatic pathophysiological processes, but have generally failed to restore functional deficits.

Putative treatment to overcome the pathophysiological symptoms of neurodegenerative diseases or injury lies in the rapidly developing field of cellular replacement strategies. The fundamental idea behind is to replace diseased or lost neuronal tissue by intracerebral cell or tissue transplantation resulting in restoration of form and function.

In the past, transplantation strategies mainly involved the transplantation of fetal tissues and considerable successes were achieved in the treatment of Parkinson's and Huntington's disease. It was effectively demonstrated that fetal transplants could replenish destroyed and lost nervous tissue by partial reconstruction and, to some extent recovery of cognitive and motor function was achieved (Bachoud-Levi *et al.*, 2000; Bjorklund and Lindvall, 2000; Lindvall, 1999). The lack of availability of donor tissue, as well as concerns regarding the purity and viability of the transplant population has led to the search for alternative sources of donor cells (Barinaga, 2000). Only recently, the discovery of pluripotent cell populations and consecutive advances in stem cell biology has offered a promising future for cell replacement therapy.

1.1 Pluripotent Embryonic Stem Cells Display Outstanding Endogenous Properties

Embryonic stem (ES) cells, firstly isolated in the early 1980's (Evans and Kaufman, 1981; Martin, 1981), combine the advantages of a permanent cell line with the pluripotential capacity

of stem cells. They allow detailed *in vitro* investigations into the course of cell lineage specification and the molecular mechanisms involved. Pluripotent embryonic stem cells represent an ideal model system to examine basic differentiation processes of neurogenesis by correlating the temporal regulation of genes and tissue morphogenesis during *in vitro* cell differentiation with normal development *in vivo*. Furthermore, stem cell-derived neural precursor cells have been shown to differentiate to neurons and glia during normal embryonic development of the brain (Brustle, 1999; Brustle *et al.*, 1999). The isolation of pluripotent embryonic stem cells thus, not only provides an inevitable source for the study of neural differentiation processes on a cellular level but embryonic stem cells also represent ideal candidates for cellular replacement strategies.

Pluripotent embryonic stem cell are derived from the inner cell mass of the blastocyst stage of embryonic development (E3.5) (Figure 1) (Evans and Kaufman, 1981; Martin, 1981). They are defined by their unlimited potential to self-renew and their ability to develop into cell types of all three primary germ layers including germ cells (Hubner *et al.*, 2003). In contrast to terminally differentiated cells of the organism, embryonic stem cells display the absence of active senescence processes, which is attributed to their high telomerase activity (Tang *et al.*, 2001; Thomson *et al.*, 1998). They can be maintained *in vitro* for prolonged periods of time in an undifferentiated state, but rely on the presence of leukæmia inhibitory factor (LIF) in culture medium and/ or the growth on mitotically inactivated mouse embryonic fibroblasts (MEF) (Smith *et al.*, 1988; Williams *et al.*, 1988). The developmental potential of embryonic stem cells has been demonstrated by the development of normal chimeras after incorporation into mouse blastocysts and *in vitro* differentiation of ES cells into a variety of endodermal, mesodermal and ectodermal derivatives (Figure 1) (Bradley *et al.*, 1984; Nagy *et al.*, 1990; Nichols *et al.*, 1990). To date, ES cell lines are available from a variety of species including rodents, rabbit, pigs, primates and human embryos (Evans and Kaufman, 1981; Graves and Moreadith, 1993; Li *et al.*, 2003; Martin, 1981; Thomson *et al.*, 1998; Thomson *et al.*, 1995; Thomson *et al.*, 1996). Since their discovery, continuous research in the field of ES cell *in vitro* expansion and differentiation shortly yielded a series of culture manipulations that promote the production of enriched populations of various cellular lineages.

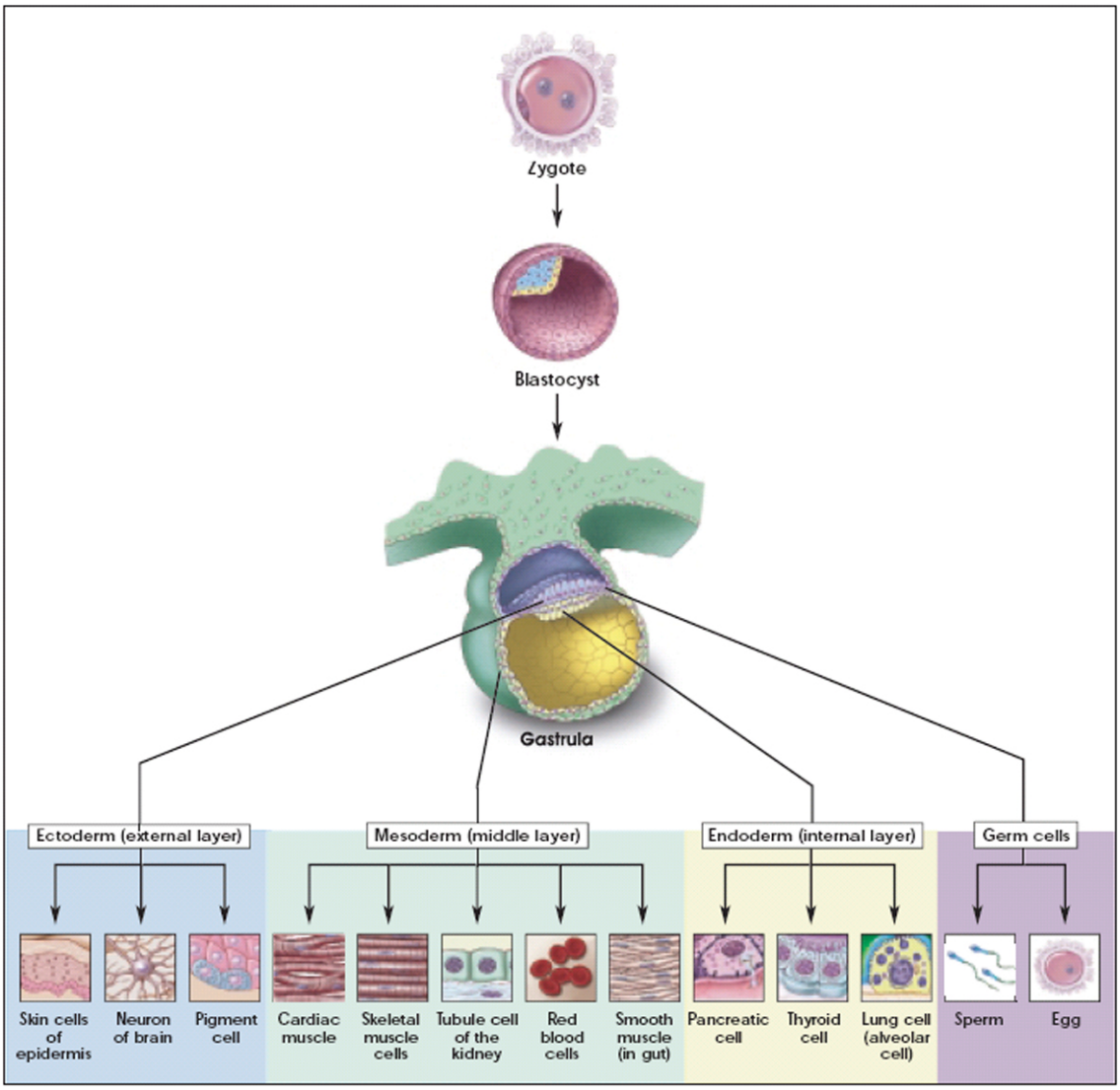


Figure 1 Lineage potential of pluripotent embryonic stem cells

Pluripotent embryonic stem cells of the blastocyst stage of embryonic development provide the basis for multilineage differentiation during gastrulation. *In vitro* ES cells can be expanded indefinitely and can be directed towards multiple cellular phenotypes including germ cells. Figure taken from *Stem Cells: Scientific Progress and Future Research Directions, 2001*

1.2 *In Vitro* Differentiation of Embryonic Stem Cells Yields Adult Cellular Lineages

The presence of LIF and/ or MEF in ES cell culture maintains ES cell's self-renewal capacity and pluripotency, possibly acting via the STAT3 pathway (Niwa *et al.*, 1998). Oct-4, a transcription factor of the POU domain, is expressed in stem cells of the early embryo maintaining their pluripotency and inhibiting lineage commitment. After gastrulation, *Oct-4* gene expression is constrained to cells of the germ lineage (Pesce and Scholer, 2000, 2001; Schoorlemmer *et al.*, 1995; Yeom *et al.*, 1996). In depth analysis of *Oct-4* gene expression patterns in differentiating stem cells revealed that, rather than acting via a simple binary on-off control system, the precise level of *Oct-4* gene expression governs distinct stem cell fates. Accordingly, a critical amount of *Oct-4* is required to sustain stem cell self-renewal, and up- or downregulation induces divergent developmental programmes. Hence, the transcription factor Oct-4 has been ascribed the role of a master regulator in pluripotent ES cells (Niwa *et al.*, 2000). Additionally, variant homeodomain containing protein Nanog, the SRY family member Sox2, Foxd3, a member of the forkhead winged-helix family and possibly Wnt signalling are also thought to play an important role in the inhibition of differentiation events (Avilion *et al.*, 2003; Hanna *et al.*, 2002; Hart *et al.*, 2004; Sato *et al.*, 2004).

Upon removal of LIF and/ or MEF from ES cell culture, ES cells *in vitro* spontaneously differentiate into a broad spectrum of cellular lineages, progressively losing their pluripotent properties (Figure 2) (Blau *et al.*, 2001; Keller, 1995). The most common method used for *in vitro* differentiation is the aggregation of ES cells into 3-dimensional 'embryoid bodies' (EBs) via 'hanging drops' (Boheler *et al.*, 2002; Doetschman *et al.*, 1985; Wobus *et al.*, 1991).

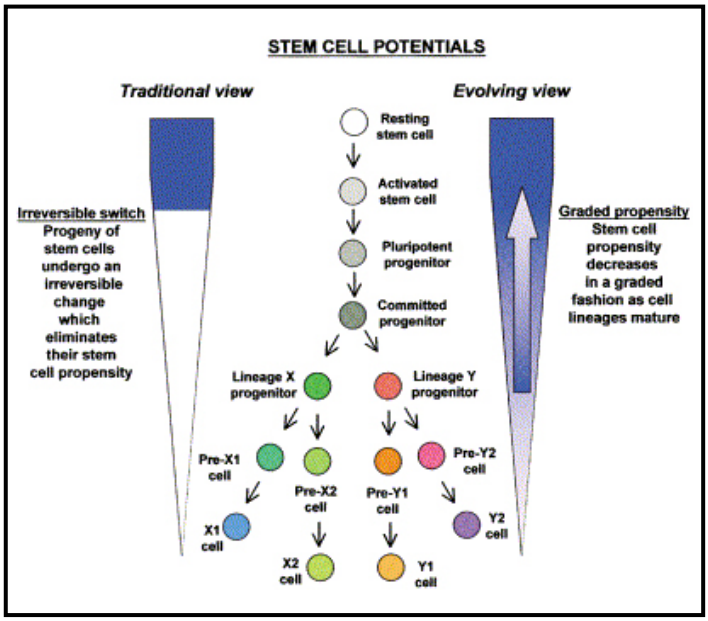


Figure 2 Two conceptual views of decreasing stem cell potential during development.

Traditionally, lineage commitment has been viewed as one-way direction; pluripotent stem cells gradually lose their stem cell potential during progressive differentiation. The evolving view on the other hand assumes that cells in general have a recruitable propensity to act as stem cells, but proposes that this tendency is declining as ES cells differentiate. Figure taken from Blau *et al.*, 2001.

During embryoid body differentiation a number of cellular lineages coexist within these spherical structures, concomitantly with the regulated expression of marker genes associated with the development of primitive endoderm (*hepatocyte nuclear factor*), ectoderm (*Pax6, Mash-1*) and mesoderm (*T brachyury*, *BMP-4*) (Wiles and Johansson, 1997, 1999). Because of the structural resemblance of embryoid bodies with the organised development of the post-implantation embryo, these cellular aggregates are often exploited to investigate the complex interactions of cellular differentiation and gene expression during early embryogenesis *in vitro* (Abe *et al.*, 1996; Itskovitz-Eldor *et al.*, 2000; Leahy *et al.*, 1999). The exposure of ES cells to various chemical agents, such as retinoic acid, dimethyl sulfoxide and 4-hydroxymethylbenzoic acid, as well as addition of growth factors and cytokines to culture medium (e.g. tumor necrosis factor-α, activin A, bone morphogenic protein-2 and-4, insulin, nerve growth factor, basic fibroblast growth factor, ciliary neurotrophic factor, triiodothyronine) has further led to the directed differentiation into tissue-specific cell types. One of the most extensively analysed differentiation paradigms comprises the spontaneous cardiac development of ES cells, firstly reported by Wobus and colleagues in 1991. Following the detection of spontaneously beating cardiomyocytes in ES cell culture, it was shown that the observed differentiation of ES cells towards terminally differentiated cardiomyocytes followed similar patterns as *in vivo* organ development (Boheler *et al.*, 2002; Hescheler *et al.*, 1997; Wobus *et al.*, 1991).

Apart from cardiac differentiation, a range of other mesodermal cells types have been obtained from ES cells including chondrocytes, osteoblasts, adipocytes as well as endothelial cells (Buttery *et al.*, 2001; Dani *et al.*, 1997; Feraud and Vittet, 2003; Kramer *et al.*, 2000). Representatives of the endodermal lineages including pancreatic islets, hepatocytes, thyrocytes, lung and intestinal cells could also be derived by *in vitro* differentiation (Ali *et al.*, 2002; Jones *et al.*, 2002; Lin *et al.*, 2003; Stoffel *et al.*, 2004; Yamada *et al.*, 2002). In view of later clinical application in neurodegenerative disease or injury paradigms, the *in vitro* differentiation of embryonic stem cells towards functional phenotypes of the neuronal lineage is of particular interest.

1.3 *In Vitro* Neuronal Differentiation Recapitulates *In Vivo* Neural Crest Development

Numerous publications describe the *in vitro* differentiation of ES cells to cells of the ectodermal lineage including neural progenitor populations as well as terminally differentiated post-mitotic neurons. *In vitro* differentiation protocols take advantage of the ability of ES cells to differentiate spontaneously into neural cell populations. This default mechanism is to some extent restricted but can be significantly improved by treatment with the vitamin A derivative retinoic acid (RA) (Bain *et al.*, 1996; Fraichard *et al.*, 1995; Strubing *et al.*, 1995; Wobus *et al.*,

1997). Molecular mechanisms of RA action during embryogenesis and in the adult brain *in vivo* are proposed to function via a complex signalling pathway, but the mechanisms whereby RA induces neural differentiation of ES cells *in vitro* are yet not fully understood. Resembling *in vivo* neurogenesis, induction of neuronal differentiation *in vitro* follows a progressive restriction from bipotential radial glial-like stem cells via lineage-determined progenitor cells to early post-mitotic and mature neurons (Figure 3) (Alvarez-Buylla *et al.*, 2001; Kempermann *et al.*, 2004). Limited mitotic activity and the developmentally regulated expression of various neural genes, transcription factors and proteins, as well as the display of differential cellular morphologies mark progressive lineage restriction (Guan *et al.*, 2001; Lang *et al.*, 2004; Rathjen and Rathjen, 2002; Stavridis and Smith, 2003). Following the aggregation of embryoid bodies and RA induction, the cellular spheres are dissociated and replated in monolayer culture. The subsequent withdrawal of serum from culture medium results in an inhibition of mesodermal lineage commitment, observable by the downregulation of mesodermal genes, such as *T brachyury*, *cardiac actin* and *cardiac globin* and the detection of a temporally controlled expression pattern of neural-specific genes (Bain *et al.*, 1996). The characteristic developmental sequence of progressing neuronal differentiation events starts with upregulation of neural transcription factors Mash-1, Wnt-1, Sonic hedgehog (Shh), Pax6 and Engrailed-1 (En-1) (Joyner, 1996; Mehler, 2002). Addition of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) yields high amounts of proliferating neural precursor cells marked by the elevated expression (> 85%) of the intermediate filament protein nestin (Lee *et al.*, 2000; Okabe *et al.*, 1996). *Nestin* mRNA expression is closely correlated with a mitotically active bipotential neural progenitor cell state in the developing central nervous system *in vivo* from which glial and neuronal cell emanate. Furthermore, in neural stem cells *nestin* gene expression has been shown to diminish upon commitment to either the oligodendroglial or neuronal lineage. (Dahlstrand *et al.*, 1995; Mellodew *et al.*, 2004). Thus, *nestin* gene expression marks a developmentally decisive position during neuronal development. Accordingly, withdrawal of bFGF and EGF from culture medium is followed by downregulation of *nestin* and *Mash-1* gene expression. Neuron-specific genes like the synaptic vesicle protein *synaptophysin*, *Nurr1* and *tyrosin hydroxlase* are in turn upregulated (Guan *et al.*, 2001; Rathjen and Rathjen, 2002).

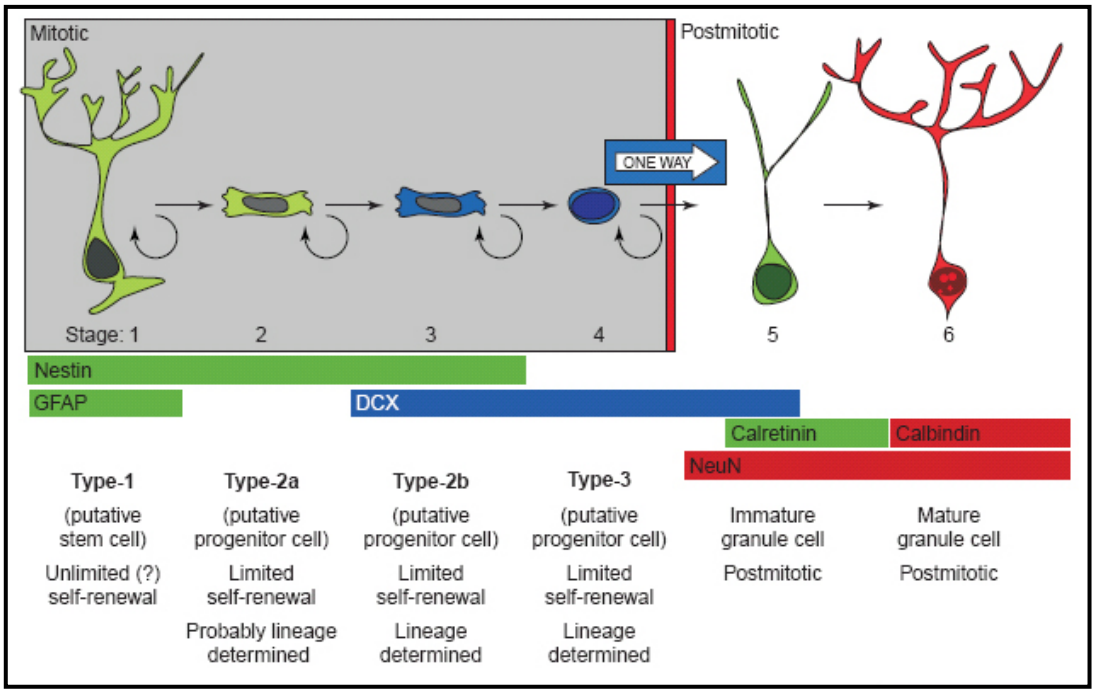


Figure 3 Six stages of progressive neuronal lineage restriction in the adult CNS

Although different hypotheses exist concerning the various stages of lineage commitment in the developing as well as in the adult nervous system, it is generally agreed on that the point of origin is a putative stem cells with radial glia and astrocytic properties. From there on lineage commitment is supposed to progress over (three) putative transiently amplifying progenitor cell stages. Different stages of development can be identified by morphological appearance, proliferative capacity and expression of stage-specific markers (e.g. Nestin, GFAP, DCX, NeuN, Calretinin and Calbindin). Hypothesis proposed by and figure taken from Kempermann *et al.*, 2004.

At an early stage of neural development genes encoding low (68 kDa) and middle (160 kDa) molecular weight *neurofilament* isoforms are expressed following neuronal lineage commitment and prior to cell cycle exit (Guan *et al.*, 2001). Mature neurons are eventually characterised by the expression of high molecular weight (200 kDa) neurofilament proteins, neurocan, syntaxin, synaptobrevin, neuronal cell adhesion molecules (NCAM), class III β-tubulin and microtubule-associated proteins MAP2 and MAP5 (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Guan *et al.*, 2001; Strubing *et al.*, 1995). Functional classification of post-mitotic neurons comprises the production of neurotransmitters dopamine, serotonin, gamma-aminobutyric acid (GABA) and glutamate, the expression of voltage-gated Ca^{2+} , Na^{+} , and K^{+} ion channels and expression of receptor-operated $GABA_A$, glycin, kainite, and N-methyl-D-aspartate (NMDA) ion channels (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Rohwedel *et al.*, 1998; Strubing *et al.*, 1995).

Gene expression and electrophysiological studies of ES cell-derived neural populations have demonstrated the presence of all three major cell types of the brain: neurons (dopaminergic, GABAergic, serotoninergic, glutaminergic or cholinergic neurons), astroglia and oligodendroglia

(Guan *et al.*, 2001; Lang *et al.*, 2004; Rathjen and Rathjen, 2002; Stavridis and Smith, 2003). Glial differentiation has been monitored by the expression of glial fibrillary acidic protein (GFAP), OP4 (for astrocytes), and GalC and O4 (for oligodendrocytes). The addition of survival promoting factors (SPF) such as interleukin-1β, dibutyryl-cAMP, transforming growth factor-β_3, glial cell-derived neurotrophic factor and neurturin to ES cell differentiation culture can further promote lineage commitment and survival of functional neurons by preventing apoptosis (Rolletschek *et al.*, 2001).

Successful *in vitro* differentiation of ES cells into functional phenotypes has consequently fostered development of cell replacement strategies. Subsequently, animal models were employed to validate stem cell differentiation *in vivo*.

1.4 Upon CNS-Transplantation Stem Cells Differentiate into Neural Phenotypes

The availability of basically unlimited numbers of ES cells and lineage-committed cell populations derived thereof has subsequently led to transplantation studies for the therapy of a variety of degenerative diseases. In animal models for cardiovascular disease, liver injury and diabetes integration of ES cell grafts with improved functional outcome could be demonstrated (Doss *et al.*, 2004). However, compared to those promising results transplantation into the CNS still imposes a major challenge.

In first transplantation efforts into naïve embryonic mouse brain, Brüstle and colleagues demonstrated the differentiation of engrafted neural precursor cells into neurons, astrocytes and oligodendrocytes, which readily integrated into telencephalic, diencephalic, and mesencephalic regions of the brain (Brustle *et al.*, 1997). A similar study by Zhang and co-workers confirmed these observations after transplantation of *in vitro* differentiated human ES cell-derived neural progenitor cells into the neonatal mouse brain (Zhang *et al.*, 2001). In adult animal models, engraftment of nestin-positive ES cell-derived precursor cells into the striatum of adult rats similarly resulted in the differentiation into various neural phenotypes, detected by expression of neuronal (Thy-1) and astroglial (GFAP) proteins (Andressen *et al.*, 2001; Arnhold *et al.*, 2000b). Long-term evaluation of stem cell-based transplantation into the brain of adult mice revealed a controlled differentiation pattern and the formation of mature neural grafts consisting of cells of all three major neural subtypes (Benninger *et al.*, 2003). These observations demonstrate that in general replacement of nervous tissue by stem cell transplantation is possible, provided the host tissue is able to supply the necessary cues to direct grafted cells to a specific fate. This is particularly true for transplantation paradigms where stem cells are grafted into neonatal CNS.

Stimulated by these successful reports, implications for disease and injury were proposed. Early experimental approaches utilised different models of myelin-deficiency. Learish and

colleagues injected ES-derived glial progenitor cells into the cerebral ventricle of myelin-deficient rats and Brüstle et al. transplanted ES-derived precursor into a rat model of human myelin disease. Subsequently, graft-derived production of myelin and efficient myelination of axons in brain and spinal cord were reported (Brustle *et al.*, 1999; Learish *et al.*, 1999). In 2002, the transplantation of undifferentiated ES cells into either chemically demyelinated rat spinal cord or likewise, into the spinal cord of myelin-deficient shiverer (*shi*/*shi*) mutant mice, led to differentiation of engrafted cells into oligodendrocytes accompanied by myelin production and remyelination of host axons (Liu *et al.*, 2000). However, the described experimental settings of transplant-mediated repair have invariably been simplistic, with transplant paradigms characterised by a clear cellular deficit, a largely non-reactive CNS environment and a lack of inflammation. Although experiments such as these are fundamental to our understanding of the behaviour and differentiation potential of stem cell populations *in vivo*, they do not account for the multifaceted pathophysiology of a neurodegenerative disease state or injury, such as traumatic brain injury, which is accompanied by a complex and reactive CNS environment.

1.5 Reactive Microenvironment of Traumatic Brain Injury Limits Stem Cell Survival

In contrast to previously mentioned disease models, traumatic brain injury represents a rather heterogeneous disease which is accompanied by a wide range of pathologies (Adams *et al.*, 2000; Davis, 2000). Traumatic brain injury is associated with a massive loss of multiple cell types due to primary mechanical tissue disruption and bleeding, and the ensuing secondary insults are the major cause of delayed brain damage. Activation of genomic, cellular and/or biochemical cascades, the development of mass lesions and superimposed systemic insults in response to traumatic brain injury additionally contribute to sustained cell necrosis, apoptosis and neurological dysfunction (Davis, 2000; Sato *et al.*, 2001). In particular, influx of immunocompetent cells facilitated by break-down of the blood brain barrier and secretion of inflammatory mediators and cytokines during the acute post-traumatic phase establish an inhospitable microenvironment detrimental for cell survival (Lenzlinger *et al.*, 2001; Morganti-Kossmann *et al.*, 1992). Over the years a variety of studies were conducted aiming at the cellular replacement of lost or damaged nervous tissue in this specific disease environment. Stem cells of different origin were transplanted into animal models of traumatic brain injury and analysed for phenotypic and functional outcome. Transplantation paradigms involving immortalised progenitor cell lines such as HiB5 (embryonic (E16) rat hippocampus), C17.2 (neonatal murine cerebellum) and MHP36 cells (E14 neuroepithelium) were shown to improve motor function and spatial learning after transplantation into the traumatically injured brain. But, studies were often accompanied by additional growth factor administration or even utilised transgenic growth factor

expressing cells for engraftment, and actual contribution of grafted cells to functional recovery was not investigated (Boockvar *et al.*, 2005; Philips *et al.*, 2001; Riess *et al.*, 2002). Similarly, the administration of bone marrow stromal cells (BMSCs) in Controlled Cortical Impact (CCI) models of traumatic brain injury led to improvement of neurological outcome, and moreover, expression of neuronal and glial markers could be observed (Lu *et al.*, 2001; Mahmood *et al.*, 2001a; Mahmood *et al.*, 2001b). Nevertheless, reports on terminal differentiation of transplanted cells were rather inconsistent, and the mechanisms of graft function were not elucidated. Following ES cell-derived neuronal and glial precursor transplantation into a Controlled Cortical Impact model of traumatic brain injury, Hoane and colleagues also reported improved functional outcome in addition to cell migration and reduction in lesion size (Hoane *et al.*, 2004). Likewise, transplantation of ES cells into lateral fluid percussion (LFP)-injured rat brain led to significant recovery of motor function (Riess, unpublished data). Compared to engraftment into the naïve CNS, the survival rate of grafted cells in a trauma environment, if determined at all, was rather low. The small number of surviving cells and, in the majority of cases, lacking evidence of terminal differentiation suggest that functional improvements were not achieved on the basis of cellular replacement (Schouten *et al.*, 2004). Alternative mechanisms of graft function involving trophic support provided by implanted cells or even stimulation of endogenous regenerative processes must thus be reconsidered. Furthermore, if differentiation of engrafted stem cells was in fact assessed in *in vivo* studies, migrating cells predominantly exhibited glial phenotypes in response to injury (Lacza *et al.*, 2003). On the one hand, these studies provide encouraging reports on a functional level, and speculations on the molecular mechanisms involved. On the other hand it was demonstrated that pathological environmental conditions critically affect intrinsic stem cell properties and thus, emphasise our lack of understanding the control of stem cell differentiation *in vivo* (Keirstead, 2001). Successful differentiation of multipotent and progenitor cells into the three major CNS cell types in standardised *in vitro* culture cannot necessarily be transferred to a complex and multifaceted *in vivo* environment. Here, post-transplantation stem cell fate largely depends on both, intrinsic features of the particular cell type and the host environment (Cao *et al.*, 2002a). Proposed stem cell-environment interactions were evidenced in two reports from 1996. Suhonen and colleagues transplanted adult hippocampal progenitors (AHPs) into neurogenic and non-neurogenic sites of the cerebellum of adult rats. In parallel, Svendsen and co-workers grafted stem cells derived from embryonic rat mesencephalon or striatum into the striatum of rats with either ibotenic acid or nigrostriatal dopamine lesions. The first group demonstrated that grafted cells would only differentiate when grafted into neurogenic, but not when transplanted into non-neurogenic regions of the brain. In contrast, the

second group showed a restricted proliferation and migration potential of grafted cells with only minor signs of differentiation when transplanted into lesioned CNS (Suhonen *et al.*, 1996; Svendsen *et al.*, 1996). Both results soundly indicate a strong impact of the encountered milieu at the site of implantation on the proliferation and differentiation capacity of engrafted cells. Moreover, Cao and co-workers showed that differentiation of neuronal-restricted precursors was inhibited when engrafted into the contused adult rat spinal cord, possibly due to the inhospitable environmental condition encountered (Cao *et al.*, 2002b). These findings support the theory that the fate of stem cells is altered in response to pathological environmental conditions. Likewise, Okano and colleagues described that the post-traumatic microenvironment of the spinal cord was in an acute inflammatory stage following injury, unfavourable for the survival and differentiation of NSC transplants (Okano *et al.*, 2003). Indeed, in a fluid percussion traumatic brain injury model, Molcanyi and co-workers demonstrated the loss of implanted stem cells due to extensive phagocytosis by activated microglia and macrophages (Molcanyi, unpublished data). Consequently, graft - environment interactions play an important role in graft survival, differentiation and integration, and finally, function (Tate *et al.*, 2002). Although lineage restriction and migration ability of a particular progenitor/ stem cell type have already been correlated with stem cell origin, type and age, considerations must be extended to include the graft-receiving host tissue. A familiar concept postulated in context with early embryonic development and known as the microenvironmental “niche” has so only recently been envisaged.

1.6 Molecular and Cellular Signals of the Environmental "Niche" Guide Cell Fate

From *in vivo* embryonic development it is known that the developmental fate of differentiating cells is largely depending on an intricate, site-specific interplay of specific regulatory genes, numerous signalling molecules and their receptory counterparts regulating the developmentally specific expression of proteins, thereby indicating different stages of development (Czyz and Wobus, 2001; Watt and Hogan, 2000). In particular, early neuronal development *in vivo* involves a sequential chain of fundamental processes that guide the way to the complex organisation of the mammalian CNS. Key molecular processes control crucial events in the life of a neuron, such as neural induction, regional and neuronal specification, migration of neurons, axonal growth and guidance and synapse formation. A precisely timed orchestration of signalling cascades and gene expression leads from neural tube segmentation through cell migration to interacting neuronal networks. Integrated regulatory mechanisms, responsible for the spatial organisation and ultimately, functional efficacy organise and manage the expression of tissue specific genes, proteins, receptors and ion channels in a developmentally controlled manner. The developmental "niche" takes part in directing programmed differentiation and is constituted of cells that, via secretion of humoral and signalling factors as well as ECM proteins, influence the developmental pathway of cells by directing their differentiation in an autocrine or paracrine manner.

In the course of *in vivo* embryonic development, transcriptional regulation during early spatial patterning of the neural tube is strongly influenced by cell-cell communications. Members of the *Drosophila* homologue segment polarity gene families *engrailed* (*en*), *wingless* (*wnt*) and *pax* functionally interact resulting in the morphogenesis and differentiation of specific brain regions, namely mesencephalon and anterior metencephalon (Joyner, 1996). The *Wnt* gene family consists of more than 10 secreted proteins that act locally via a yet unknown pathway but it is suggested that *Wnt-1* represents the signal for *En* induction. Pax action involves cell-cell signalling and the expression of all *Pax* genes (except *Pax1* and *Pax9*) is spatially and temporally restricted during CNS development (Gruss and Walther, 1992). The secreted protein of Sonic hedgehog (Shh) that was also first identified in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980) represents another extracellular signalling mechanism. Initially expressed in the axial mesoderm, it can be found in the medial neural plate and the ventral neural tube, playing an important role in the patterning of face and ventral forebrain.

Throughout development, neuronal plasticity is restricted via progressive neuronal specification; primary neurogenesis is followed by neuronal migration and the formation of synaptic interactions. Again, studies in invertebrates paved the way for elucidating mammalian

cell specification processes. In *Drosophila*, neuronal fate largely depends on intercellular signalling processes. The *Notch* family of genes encode large proteins that contain segments in the membranes of cells. These proteins act as receptors for extracellular ligands that specify cell fate, leading to tissue organisation during development. The inhibition of Notch signalling promotes neuronal differentiation, whereas activation of the transmembrane receptor Notch results in a non-neural fate of cells (Marnellos *et al.*, 2000). An interaction of cytoplasmatic proteins Notch-1 and Numb, located at opposite sites of ventricular epithelial cells has been proposed to decide on symmetric or asymmetric cellular division thereby directing cell fate (Figure 4) (Jan and Jan, 1998; Yoon and Gaiano, 2005). These perceptions fortify that instructive and restrictive cues are provided by the cellular and humoral environment and are able via intracellular signalling cascades to either potentiate maintenance and/ or proliferation of cell populations or alternatively, promote the commitment and maturation towards a specific lineage (Mehler, 2002; Mehler *et al.*, 2000).

An extraordinary example for the influence of site-specific extracellular cues is the guidance of axons by chemoattractants that are secreted by the target under the influence of boundary regions that separate specific domains of the developing CNS (Wilson *et al.*, 1993). These cues can either be repulsive or attractive, acting over long or short distances. It is assumed that gradients of different molecules are used for the establishment of neuron specification, neuron guidance and interactions between neurons. Diffusible factors secreted by axonal targets such as Netrin-1 protein and semaphorins also exert an important influence (Placzek *et al.*, 1990). While Netrin-1 is a long-range chemoattractant, semaphorins are involved in the repellence of axon growth cones (Keynes and Cook, 1995; Raper, 2000; Serafini *et al.*, 1996). Recently detected Slit proteins do also play a role in axon guidance and cell migration (Brose and Tessier-Lavigne, 2000).

Another example of secreted molecules that play a major role in neuronal development and post-neurotraumatic injury are the neurotrophins. The neurotrophin family consists of a functionally and structurally related class of neurotrophic factors that provide trophic support for neurons during development and adult life. The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Levi-Montalcini, 1987). Neurotrophic factors mediate their cellular effects through the activation of protein tyrosine kinase (trk) via binding of the trk receptor family, namely trkA for NGF, trkB for BDNF and trkC for NT-3. This class of polypeptides is involved in the differentiation, function and survival of neuronal cell populations and synthesised by neurons in the adult rat brain, particularly in the hippocampus (Barbacid, 1995a, b; Barde, 1990; Klein, 1994; Thoenen,

1991; Vantini, 1992). In animal models of brain injury, the administration of neurotrophic factors via injection or via transplantation of cells genetically engineered to secrete neurotrophins has been shown to promote neuronal survival and repair by re-establishing functional connections and promoting the sprouting of neurits and the guidance of axons. In addition, neurotrophins delay apoptosis, prevent atrophy of axotomised neurons and enhance the expression of growth-associated genes. The neuroprotective and regenerative capacities of neurotrophic factors make them ideal candidates for injury-related applications and only lately, this protein family has been proposed a major role in stem cell-based functional recovery (Chen *et al.*, 2005; Chen *et al.*, 2002b; Lu *et al.*, 2003; Mocchetti and Wrathall, 1995).

Recapitulating, a complex regulatory network that utilises cooperative, complementary, sequential and antagonistic extrinsic and intrinsic regulatory mechanisms is required to

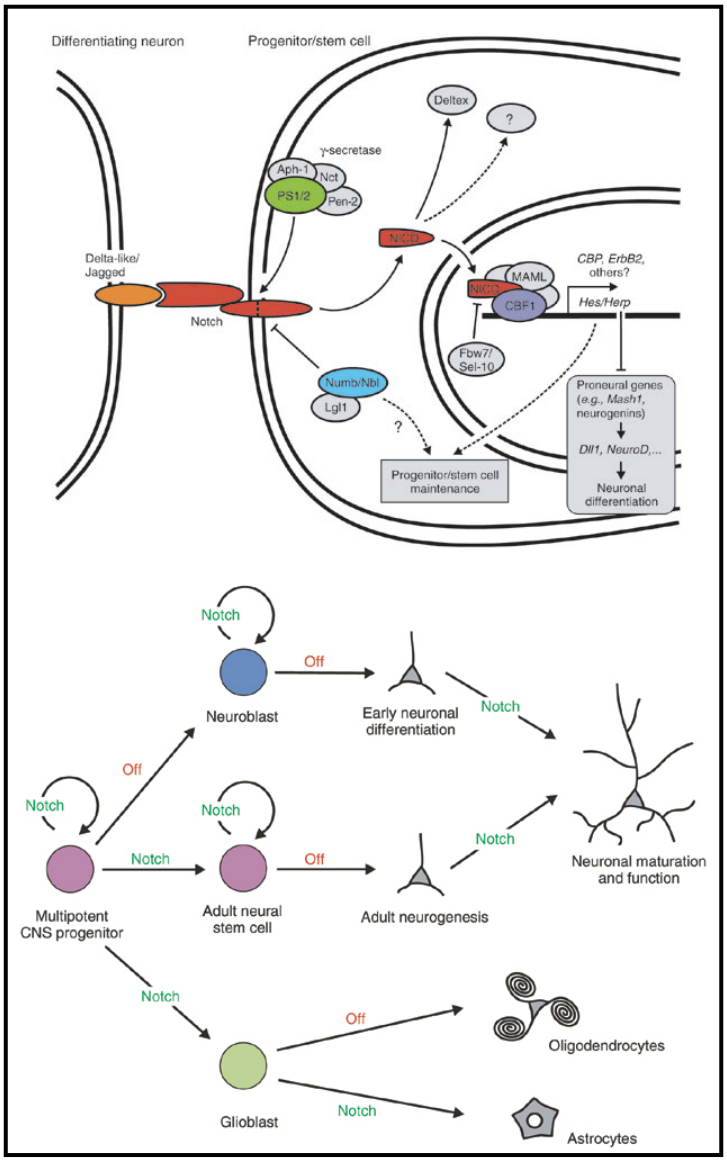


Figure 4 Intercellular communication via Notch signalling generates lineage diversity

Within the developing and adult CNS Delta/ Notch interactions keep the balance between different modes of cell division, symmetric vs. asymmetric. Adjacent cells communicate via Delta/ Notch interaction determining their cell fate. Binding of Notch transmembrane receptor to its soluble ligand Delta leads to a cascade of intracellular events that antagonise neuronal gene expression and consequently, inhibit differentiation.

Differentiating neurons activate Notch signalling in adjacent cells to guarantee preservation of a pool of multipotent CNS progenitor cells. Notch signalling is generally involved in the maintenance of a multipotent state and later on in development promotes neuronal maturation and function. However, downregulation of Notch signalling is essential for the initiation of neurogenesis (Notch: Notch signalling is possibly involved; Off: downregulation of Notch is necessary). Both figures were taken from Yoon and Gaiano, 2005.

orchestrate the evolving profiles of lineage species during embryonic development. A minor shift in the character of a growth factor, signalling molecule concentration or ECM protein distribution constituting the developmental niche may therefore regulate and change depending cell fates. Factors and mechanisms responsible for developmental plasticity are still active in neonatal organs and especially in germinal centers of the adult organism where the composition of different cell types and their functional interaction has been termed "stem cell niche".

1.7 Stem Cell Niche Controls Self-Renewal and Lineage Commitment

With regard to embryonic development the concept of the "niche" has been postulated and was re-introduced regarding the plasticity of adult stem cells (Schofield, 1983; Watt and Hogan, 2000). Adult stem cells can be found in a variety of adult tissues, some of them are known to undergo very limited regeneration or turnover, including the epidermis, skeletal muscle, bone marrow and the nervous system (Prockop *et al.*, 2003; Watt and Hogan, 2000). Areas of neurogenetic activity were also found throughout the adult mammalian brain, especially in the hippocampus, the striatum and septum, as well as in the olfactory bulb and the subventricular zone (SVZ) (Bottai *et al.*, 2003; Gage, 2000; Galli *et al.*, 2003). Here, neural stem cells reside in close community with astroglial and microvascular endothelial cells, as well as with mature neurons of the CNS (Figure 5) (Doetsch, 2003; Palmer *et al.*, 2000; Wurmser *et al.*, 2004). The residing neural stem cells (NSC) are generally defined as mitotically active cells that migrate along restricted pathways and have the ability to differentiate into mature neurons (Bottai *et al.*, 2003; Gage, 2000; Galli *et al.*, 2003). *In vitro* these cells retain their ability to self-renew, but in contrast to embryonic stem cells they can only generate lineage-restricted progeny (Czyz *et al.*, 2003; Gritti *et al.*, 2002). Maintenance of NSC plasticity *in vivo* is the pivotal role of the so-called stem cell niche and involves a variety of cell intrinsic as well as extrinsic mechanisms. Numerous growth factor signals and cytokines regulate the proliferation, survival, migration and differentiation of NSCs and emerging progenitor cells (Figure 6) (Hagg, 2005).

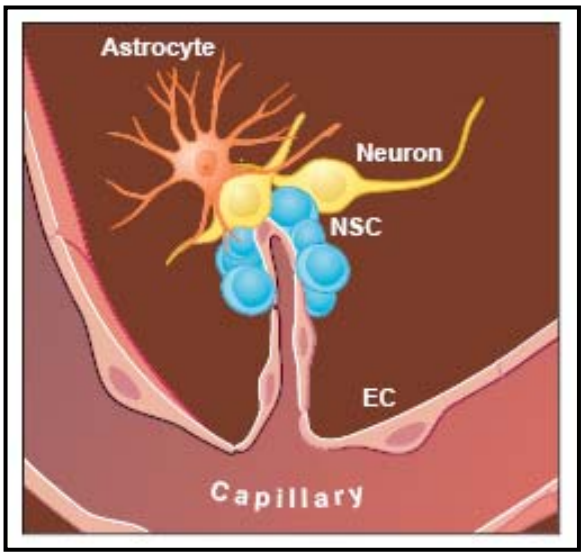


Figure 5 Stem cell niche of the adult brain

In the adult CNS, neural stem cells (NSC) reside within distinct cellular compartments close to blood vessels. Astrocytes, microvascular endothelial cells and mature neurons contribute to these so-called stem cell niches. Intercellular communication maintains the generative stem cell pool and regulates stem cell differentiation. Figure taken from Wurmser *et al.*, 2004.

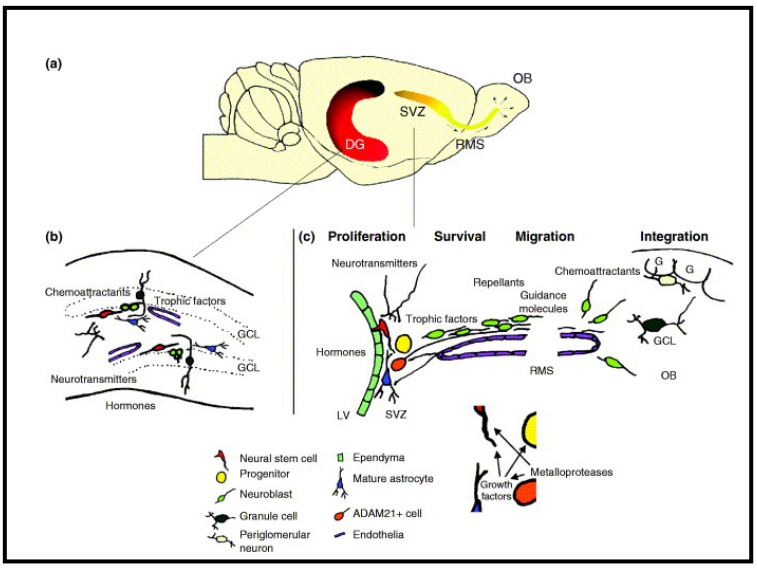


Figure 6 Neurogenesis in the adult CNS
Within the neurogenic niches of the adult CNS, molecular and cellular signals collectively regulate, coordinate and control neurogenesis from neural stem cells. Overlapping signalling pathways involve e.g. growth factors, neurotransmitters, guidance factors, metalloproteases and hormones. (a) Neurogenic niches of the dentate gyrus (DG) and forebrain. (b) In the dentate gyrus, new neurons are born in the subgranular zone just inside the granule cell layer (GCL). (c) Newly born neurons of the subventricular zone (SVZ) migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB). (G: glomeruli of the olfactory bulb; LV: lateral ventricle).
Figure taken from Hagg, 2005.

In subsequent years, many *in vitro* studies into the nature of the stem cell niche and its influence on NSCs were conducted. So the site-specific migration, functional integration and differentiation of grafted NSCs was reported, proclaiming that NSCs responsiveness to environmental cues was restricted to their site of origin (Herrera *et al.*, 2001). Moreover, it has been suggested that cues for migration and differentiation are dependent on recipient age, so that adult in contrast to fetal tissue is no longer able to provide the appropriate cues necessary for migration and differentiation of transplanted NSCs (Lim *et al.*, 1997). In contrast, data by Shihabuddin and co-workers indicated that clonally expanded, multipotent adult progenitor cells from a non-neurogenic region were not lineage-restricted to their developmental origin but were able to generate region-specific neurons *in vivo* when exposed to the appropriate environmental cues (Shihabuddin *et al.*, 2000). In a pilot *in vitro* investigation into the regional specification of neural stem cell, Hitoshi and colleagues confirmed that stem cells derived from different neurogenic sites of the brain, namely embryonic cortex, ganglionic eminence and midbrain/hindbrain, express separate molecular markers of regional identity *in vitro*. But, in the same study it was also shown that NSC's regional identity could be altered by local inductive cues as observed in organotypic slice cultures (Hitoshi *et al.*, 2002). The important role of extrinsic signals regulating intrinsic properties of neural progenitor cells was supported by Lillien and Raphael 2000 who related temporal changes during development to cell-cell signalling events within the developmental niche (Lillien and Raphael, 2000). Closer investigation into cell-cell communication within specific stem cell niches revealed that adjacent non-stem cells exerted significant influence on residing stem cell fate. It could be shown that ES cell-derived motoneurons extended long axons, formed neuromuscular junctions and induced

muscle contraction when co-cultured with myoblasts (Harper *et al.*, 2004). Astrocyte co-culture boosted differentiation of multipotential adult progenitor cells derived from bone marrow to a neuronal phenotype (Jiang *et al.*, 2003), and in the vascular niche endothelial cells released soluble factors that maintained CNS stem cell self-renewal and neurogenic potential (Palmer *et al.*, 2000).

Above-mentioned studies demonstrate the inductive role of the humoral and cellular environment, but *in vitro* investigations in the past primarily focussed on the differentiation control of neural stem cells and depicted mainly naïve environmental milieus. However, embryonic stem cells are, even more than adult stem cells, particularly susceptible to microenvironmental cues. In clinical applications where transplantation of neural stem cells is not favourable because of the loss or damage of multiple cell types (e.g. following traumatic brain injury), engraftment of embryonic stem cells due to their pluripotent capacities is the method of choice. In an adult organism, ES cells are exposed to environmental conditions, which are only hypothesised to direct ES cell fate in an appropriate manner. To complicate matters, ES cells are placed in dysfunctional disease or injury environments. Paradoxically, the interaction of the transplant with the host necessary for the graft to mature and function can then also be detrimental for graft survival. This may be particular true for the clinical syndrome of a traumatic brain injury where some of the hallmarks of the pathophysiology exert deleterious effects on stem cell populations following transplantation. However, graft survival and function are crucial for the therapeutic efficacy of ES cell engraftment into the CNS. Thus, *in vitro* modelling of embryonic stem cell - environment interactions under naïve and disease conditions and investigating the presumably resulting alterations in stem cell plasticity are essential for the evaluation and improvement of cell replacement strategies.

1.8 Aim of this Thesis

The rapid evolution of fresh insights into stem cell dynamics, better and faster procedures for lineage selection and thus, for the production of viable cells for transplantation has boosted the emergence of novel transplantation strategies. Cellular transplantation as a therapeutic option in the treatment of traumatic brain injury has only recently begun to be explored with promising behavioural results. In a variety of transplant studies however, behavioural efficacy or some recovery of function has been observed in the absence of (or prior to) the terminal differentiation of grafted cells. In those studies that document actually proper terminal differentiation, the number of differentiated cells is often small compared to those lost to disease or injury-induced cell death (Schouten *et al.*, 2004). Thus, it becomes apparent that even though stem cells of diverse origin have been shown to differentiate into the three major CNS cell types *in vitro*, their

fate after transplantation in the adult brain largely depends on the host environment. Understanding both, the extrinsic signals that recruit and direct stem cell fate *in vivo* and the endogenous properties that limit the ability of a stem cell to respond to a given set of conditions is essential for the development of more effective therapeutic approaches to CNS injury.

Introducing a sophisticated *in vitro* co-culture model, this doctoral thesis elucidates the effect of exogenous biological activities on stem cell plasticity by modelling naïve and pathological environment - stem cell interactions. In a first experimental approach, murine ES cells were co-cultured with endothelial and astroglial cells that contribute to the cerebral stem cell niche *in vivo*. After a co-culture period of 7 days, ES cells were examined for expression of early neuronal lineage markers by RT-PCR. Utilising differential co-culture settings cell contact and/ or humorally mediated environmental influences on stem cell self-renewal and differentiation potential could be distinguished.

In a second model system, the impact of environmental conditions associated with the aetiology of traumatic brain injury on stem cell properties was investigated. Using a fluid percussion device traumatic brain injury was inflicted on rodent brains (McIntosh *et al.*, 1989). Subsequent processing of respective brains yielded an enriched brain tissue extract. ES cells were grown in the presence of this extract for up to 10 days. During this time, ES cell's mitotic activity was assessed by flow cytometry. Additionally, ES cells were harvested at various time points and analysed for the expression of early ectodermal, endodermal and mesodermal lineage markers, as well as for the expression of neuronal differentiation markers. Simultaneously, the ability of ES cells to secrete neuroprotective proteins of the neurotrophin family (BDNF, NGF, NT-3) into the culture medium upon treatment with brain tissue extract was examined by ELISA. Hence, this doctoral thesis provides experimental data on i) interactions between ES cells and endothelial/ glial cells belonging to the neural stem cell niche *in vivo*, ii) the impact of traumatic brain injury-related parameters on stem cell plasticity and, iii) this study elucidates the source of beneficial effects observed after stem cell transplantation into animal models of traumatic brain injury.

2 MATERIALS & METHODS

2.1 Chemicals and Proteins

7-amino-actinomycin D (7-AAD)	BD Biosciences, Heidelberg, DE
Accutase II	PAA Laboratories GmbH, Cölbe, DE
Agarose, electrophoresis grade	Invitrogen GmbH, Karlsruhe, DE
Albumin, bovine (BSA)	Serva Electrophoresis GmbH, Heidelberg, DE
Ammonium chloride	Merck KGaA, Darmstadt, DE
Aqua ad iniectabilia	DeltaSelect GmbH, Dreieich, DE
Collagenase/ dispase	Roche Diagnostics GmbH, Mannheim, DE
Dimethylsulfoxid (DMSO)	PeqLab Biotechnologie GmbH, Erlangen, DE
Dulbecco's buffer substance	Serva Electrophoresis GmbH, Heidelberg, DE
Ethanol	Carl Roth GmbH & Co. KG, Karlsruhe, DE
Formaldehyde	Merck KGaA, Darmstadt, DE
Glacial acetic acid	J.T. Baker/ Mallinckrodt Baker, Griesheim, DE
Hydrogen chloride	Merck KGaA, Darmstadt, DE
Methanol	Carl Roth GmbH & Co, Karlsruhe, DE
Normal goat serum	DakoCytomation GmbH, Hamburg, DE
Paraformaldehyde	J.T. Baker/ Mallinckrodt Baker, Griesheim, DE
Pentobarbital sodium	Merial GmbH, Halbermoos, DE
Percoll	Roche Diagnostics GmbH, Mannheim, DE
Picric acid	Merck KGaA, Darmstadt, DE
Potassium chloride (KCl)	Merck KGaA, Darmstadt, DE
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, DE
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt, DE
TE buffer	Invitrogen GmbH, Karlsruhe, DE
Tris(hydroxymethyl)aminomethane	Merck KGaA, Darmstadt, DE
Triton x-100	Merck KGaA, Darmstadt, DE
Trypan blue	Biochrom AG, Berlin, DE
Trypsin/ EDTA	Biochrom AG, Berlin, DE
Ultra-pure water (ddH_2O)	Biochrom AG, Berlin, DE
UltraMount mounting medium	DakoCytomation GmbH, Hamburg, DE

All other chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH, Munich, DE unless stated otherwise in the text.

2.2 Equipment and Software

ABI Prism 7700 Sequence Detector	Applied Biosystems, Darmstadt, DE
Adventurer™ Precision Balances	Ohaus GmbH, Giessen, DE
Beckman centrifuge Model J-21B	Beckman Instruments, Fullerton, CA, USA
BX-40 phase contrast microscope	Olympus GmbH, Hamburg, DE
Centrifuge 5417 R	Eppendorf AG, Hamburg, DE
Jouan incubator	Thermo Electron Corp., Dreieich, DE
EasyCast™ Mini Gel Electrophoresis	Owl Separation Systems, Portsmouth, NH, USA
Eclipse E400 fluorescence microscope Incl. Lucia G Imaging Software	Nikon GmbH, Düsseldorf, DE
CCD-1300CB camera	VDS Vosskühler GmbH, Osnabrück, DE
Eclipse TS100 inverted microscope	Nikon GmbH, Düsseldorf, DE
Coolpix 5000 Digital Camera	Nikon GmbH, Düsseldorf, DE
Electrophoresis power supply E452	Consort nv, Turnhout, BE
FACScan incl. CellQuest Software	BD Biosciences, Heidelberg, DE
Gel-Pro® Analyzer Imaging Software	Media Cybernetics Inc., Silver Spring, MD, USA
Heraeus® Biofuge pico	Kendro Lab. Products GmbH, Langenselbold, DE
Heraeus® HERAcell® CO_2-Incubator	Kendro Lab. Products GmbH, Langenselbold, DE
Heraeus® HERAfreeze® SI-Freezer	Kendro Lab. Products GmbH, Langenselbold, DE
Heraeus® Multifuge 3 S-R	Kendro Lab. Products GmbH, Langenselbold, DE
Holten Laminar Flow Workbench	Heto-Holten GmbH, Wettenberg, DE
InoLab® pH 730 pH meter	WTW GmbH, Weilheim, DE
KSG 32-2-3 laboratory autoclave	KSG Sterilisatoren GmbH, Bottrop, DE
Rotating shaker	VWR International GmbH, Darmstadt, DE
Spectra microplate reader	Tecan Deutschland GmbH, Crailsheim, DE
Tgradient thermocycler	Biometra GmbH i.L., Göttingen, DE
Transwell® Polyester Filter Inserts	Corning B.V Life Sciences, Schiphol-Rijk, NL
Tpersonal thermocycler	Biometra GmbH i.L., Göttingen, DE
Ultra sound homogeniser	VWR International GmbH, Darmstadt, DE
Ultrospec Plus Spectrophotometer	Amersham Pharmacia Biotech, Uppsala, SE
UV-Transilluminator TFX 20M	Vilber Lourmat, Marne-la-Vallée, FR
Vortex Type Reax Top	Heidolph GmbH & Co. KG, Schwabach, DE
Waterbath, Ecoline 019 + Heating Circulator E100	Lauda GmbH & Co.KG, Lauda-Königshofen, DE

2.3 Kits and Consumables

100 bp DNA ladder plus	PeqLab Biotechnologie GMBH, Erlangen, DE
Advantage™ RT-for-PCR Kit	BD Biosciences, Heidelberg, DE
peqGOLD dNTP-Mix	PeqLab Biotechnologie GMBH, Erlangen, DE
FITC BrdU Flow Kit	BD Biosciences, Heidelberg, DE
Human BDNF Quantikine ELISA Kit	R&D Systems GmbH, Wiesbaden, DE
Loading dye (6x)	PeqLab Biotechnologie GMBH, Erlangen, DE
Nerve Growth Factor ELISA	Chemicon International Ltd., Chandlers Ford, UK
NT-3 Emax® ImmunoAssay Systems	Promega GmbH, Mannheim, DE
QIAshredder Spin Columns	Qiagen GmbH, Hilden, DE
Platinum® *Taq* DNA Polymerase	Invitrogen GmbH, Karlsruhe, DE
RNeasy Mini Kit	Qiagen GmbH, Hilden, DE
SuperScript™ One-Step RT-PCR Kit With Platinum® *Taq*	Invitrogen GmbH, Karlsruhe, DE
*Taq*Man® Universal PCR Master Mix, No AmpErase® UNG	Applied Biosystems, Darmstadt, DE
TrackIt™ 50 bp DNA ladder	Invitrogen GmbH, Karlsruhe, DE
Vectastain® ABC Kit	Vector Laboratories, Burlingame, CA, USA

All disposable plastic ware required for tissue culture and molecular biological applications like 15 ml and 50 ml Falcon tubes, 6-, 12-, and 96-well plates, petri dishes and cell culture flasks in various sizes, PCR tubes and sterile pipette tips were purchased from Greiner Bio-One GmbH, Solingen-Wald, DE and Sarstedt AG & Co., Nümbrecht, DE unless mentioned otherwise.

2.4 Buffers and Solutions

Most standard buffers and solutions like PBS, TAE, TBS, DNA loading buffer and RNase-free water were prepared according to Sambrook *et al.* 1989, using ddH_2O. When necessary, buffers and solutions were either autoclaved for 25 min at 121°C in a KSG 32-2-3 laboratory autoclave or filter sterilised (0.22 µm pore size). Adjustments of pH values were carried out at RT using HCl or NaOH and an InoLab® pH 730 pH meter.

Cell Culture

Freezing medium	20% FCS
	10% DMSO
	20% PBS (w Ca^{2+} Mg^{2+})
	50% appropriate culture medium
33% Percoll gradient	19 mL PBS (1x)
	1 mL PBS (10x)
	1 mL FCS
	10 mL Percoll
	Centrifugated for 60 min at 30,000 x *g* (4°C)

RNA Gel Electrophoresis

1.2% FA agarose gel	0.6 g agarose
	5 mL 10x FA gel buffer
	Ad 50 mL RNase-free water
	0.9 mL of 37% (12.3 M) formaldehyde
10x FA gel buffer	200 mM MOPS
	50 mM sodium acetate
	10 mM EDTA
	Adjusted to pH 7.0
1x FA running buffer	500 mL FA gel buffer (10x)
	10 mL 37% (12.3 M) formaldehyde
	440 mL RNase-free water
5x RNA loading buffer	8 µL saturated aqueous bromophenol blue solution
	40 µL 500 mM EDTA, pH 8.0
	360 µL 37% (12.3 M) formaldehyde
	1 mL 100% glycerol
	1542 µL formamide
	2 mL FA gel buffer (10x)
	50 µL RNase-free water

Immunocytochemistry	
FixA	4% paraformaldehyde
	0.2% picric acid
	1% glutardialdehyde
	2% sucrose
	0.1 M sodium acetate
	Adjusted to pH 6.0
Flow Cytometry	
1x DPBS	0.2 g KCl
	0.2 g KH_2PO_4
	8.0 g NaCl
	2.16 g $Na_2HPO_4 \cdot 7\ H_2O$ (pH 7.2-7.4)
	Ad 1 L ddH_2O
Staining buffer	1x DPBS
	3% FCS
	0.09% sodium azide

2.5 General Cell Culture Conditions and Media

2.5.1 Culture Conditions

All cells and cell lines used in this study (Table 1) were cultivated in Heraeus® CO_2-Incubators at 37°C in a humidified atmosphere containing 5% CO_2. Cells were supplied with fresh medium every other day and passaged upon ~ 90% confluence. Cell culture techniques were carried out aseptically under a Holten Laminar Flow Workbench. For storage, cells were resuspended in freezing medium and kept in liquid nitrogen. Cell counts were performed with a haemocytometer (Neubauer chamber, VWR international GmbH, Darmstadt, DE) under a BX-40 phase contrast microscope at 1,000 x magnification. Percentage of viable cells was evaluated by trypan blue exclusion. All cell culture media and supplements were purchased from Biochrom AG, Berlin, DE unless mentioned otherwise.

Table 1 Primary cells and cell lines used

Name	Description	Species	Reference
ASPC-1	Adenocarcinoma	human	ATCC, Manassas, VA, USA: CRL-1682
BAC7	GFP-transfected (under control of a β-actin promoter) embryonic stem cells (derived from ES-D3)	mouse	Courtesy of Prof. Dr. J. Hescheler, Centre of Physiology and Pathophysiology, Institute for Neurophysiology, Medical Faculty, University of Cologne, DE
Feeder cells	Embryonic fibroblasts (E14)	mouse	Same as above
b.End5	Brain endothelioma	mouse	ECACC, Salisbury, UK: 96091930
C6	Glial tumour	rat	ECACC, Salisbury, UK: 92090409
CGR8	Embryonic stem cells	mouse	Courtesy of Prof. Dr. A. Sachinidis, Centre for Physiology and Pathophysiology, Institute for Neurophysiology, Medical Faculty, University of Cologne, DE
HUVEC-c	Umbilical vein endothelial cells	human	PromoCell GmbH, Heidelberg, DE
MHEC-5T	Heart endothelial cells	mouse	DSMZ, Braunschweig, DE: ACC 336
Neuro-2a	Neuroblastoma	mouse (albino)	Courtesy of Dr. C. Clemen, Institute for Biochemistry I, Medical Faculty, University of Cologne, DE
PC-12	Pheochromocytoma	rat	Same as above
pRBEC	Primary cerebral microvascular endothelial cells	rat	Isolated from 14 day old Wistar rats
SV-ARBEC	SV40-transfected brain microvascular endothelial cells	rat	Courtesy of Dr. A. Muruganandum, Institute for Biological Sciences, National Research Council of Canada (NRC), Ottawa, CAN

2.5.2 General Culture Media

ASPC...

...RPMI 1640 medium (1x) w stable glutamine + 10% FCS + 1% penicillin/ streptomycin solution (ScienCell Research Laboratories, San Diego, CA, USA)

BAC7...

...Dulbecco's Modified Eagle Medium (DMEM) (1x) w stable glutamine + 5 mM sodium pyruvate + 15% FCS + 50 µg/mL gentamycin + 0.5 µg/ml patricin + 1x non-essential amino acids + 100 µM β-2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Munich, DE) + 1,000 units/mL leukæmia inhibitory factor (LIF) (Chemicon International Ltd., Chandlers Ford, UK) + 0.4 mg/mL geniticin (G418) (Invitrogen GmbH, Karlsruhe, DE)

Cultivation: cells were grown on a feeder layer of mitotically inactivated (mitomycin-treated) mouse embryonic (E14) fibroblasts (MEF).

b.End5, MHEC-5T...

...DMEM (1x) w stable glutamine + 5 mM sodium pyruvate + 10% FCS + 50 µg/mL gentamycin + 0.5 µg/mL patricin

C6...

...Ham's F-12 medium (1x) w stable glutamine + 5 mM sodium pyruvate + 10% FCS + 50 µg/mL gentamycin + 0.5 µg/mL patricin

CGR8...

...DMEM (1x) w stable glutamine + 5 mM sodium pyruvate + 10% FCS + 100 units/mL LIF + 50 µM β-2-mercaptoethanol

Cultivation: cells were grown in gelatine-coated (0.2 %) cell culture flasks.

Feeder cells (MEF)...

...DMEM (1x) w stable glutamine + 5 mM sodium pyruvate + 15% FCS + 50 µg/mL gentamycin + 0.5 µg/ml patricin + 1x non-essential amino acids + 100 µM β-2-mercaptoethanol

Mitomycin-treatment: upon confluence, medium was removed and cells were treated with culture medium supplemented with 20% mitomycin C (Serva Electrophoresis GmbH,

Heidelberg, DE) for 2 hours. Thereafter, mitomycin solution was removed from cultures, cells were washed with PBS w Ca^{2+} + Mg^{2+} and regular medium was again added to culture flasks.

HUVEC-c...

...Clonetics® EBM® Endothelial Cell Basal Medium + SingleQuot® Kit (both Cambrex Bio Science Verviers, S.p.r.l, Verviers, BE) + 10% FCS

Neuro-2a...

...DMEM (1x) w stable glutamine + 10% FCS + 1% penicillin/ streptomycin solution + 1x non-essential amino acids

Neuronal differentiation: in order to differentiate Neuro-2a cells, FCS was reduced to 0.2% in culture medium.

PC-12...

...RPMI 1640 medium (1x) w stable glutamine + 10% heat inactivated horse serum + 5% FCS + 1% penicillin/ streptomycin solution

Neuronal differentiation: 50 ng/mL nerve growth factor (NGF-7S) (Sigma-Aldrich Chemie GmbH, Munich, DE) was added to culture medium and cells were grown on poly-L-lysine (Sigma-Aldrich Chemie GmbH, Munich, DE) coated plastic ware.

pRBEC...

...DMEM (1x) w stable glutamine + 5 mM sodium pyruvate + 20% FCS + 50 µg/mL gentamycin + 0.5 µg/mL patricin + 100µg/mL heparin-sodium (Ratiopharm GmbH, Ulm, DE) + 1 ng/mL basic fibroblast growth factor (bFGF) (Sigma-Aldrich Chemie GmbH, Munich, DE)

Cultivation: cells were grown on 1% fibronectin/ collagen IV (Sigma-Aldrich Chemie GmbH, Munich, DE).

SV-ARBEC...

...Medium 199 Earle (1x) + 2 mM glutamine + 5 mM sodium pyruvate + 10% FCS + 50 µg/mL gentamycin + 0.5 µg/mL patricin + 5 mg/mL peptone (Invitrogen GmbH, Karlsruhe, DE) + 0.9% D-glucose + 1x non-essential amino acids + 1x Basal Medium Eagle (BME) vitamins

Neuronal Differentiation Media

DMEM 10%...

...100 mL DMEM (1x) w stable glutamine + 5 mM sodium pyruvate + 10% FCS + 1x non-essential amino acids + 100 µM β-2-mercaptoethanol + 40 µg/mL gentamycin + 1,000 units/mL LIF + 0.4 mg/mL G418

Iscove's 20%...

...100 mL Iscove's Modified Dulbecco's Medium + 20% FCS + 100 µM β-2-mercaptoethanol + 40 µg/mL gentamycin

B1 medium...

...DMEM/ Ham's F-12 (1:1) (1x) medium + 40 µg/mL gentamycin + 5 µg/mL insulin + 30 nM sodium selenite + 5 µg/mL fibronectin + 50 µg/mL transferrin (all Sigma-Aldrich Chemie GmbH, Munich, DE)

B2 medium...

...Neurobasal™ Medium + 4 mL B27 supplement (both Invitrogen GmbH, Karlsruhe, DE) + 5% FCS + 40 µg/mL gentamycin + 20 nM progesterone + 100 µM putrescine + 10 mg/mL insulin + 1 µg/mL laminin + 30 nM sodium selenite + 50 µg/mL transferrin (all Sigma-Aldrich Chemie GmbH, Munich, DE)

Conditioned Medium

Conditioned medium was prepared from confluent cell cultures incubated with appropriate cell culture medium for 48 hours. Thereafter, medium was withdrawn from cell culture, filter-sterilised and diluted 1:2 in serum-free DMEM/Ham's F-12.

2.5.3 Neuronal Differentiation of Embryonic Stem Cells

The ES-D3 murine embryonic stem cell clone BAC7 is transfected with green fluorescent protein (GFP) under control of a β-actin promoter and can be differentiated to neuronal precursor cells (Figure 7) according to the protocol published by Arnhold *et al*. 2000b. In brief, BAC7 stem cells were seeded onto MEF feeder layer and grown for 3 - 4 days in fully supplemented BAC7 medium.

Embryoid Body (EB) Formation via "Hanging Drops"

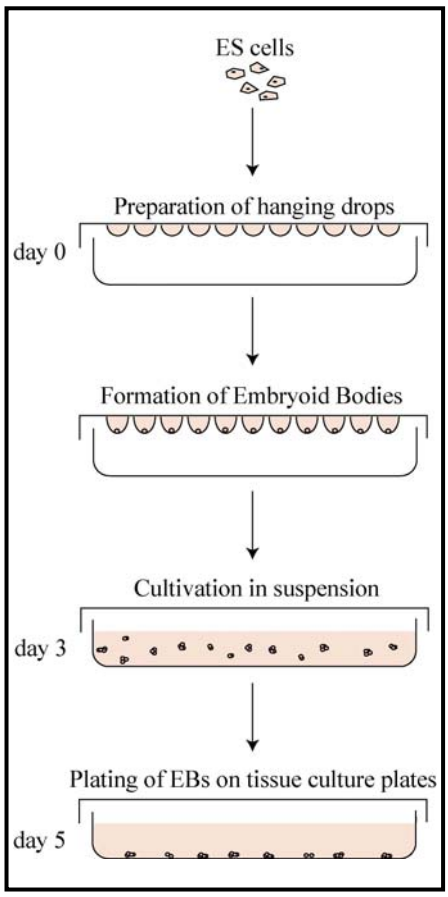


Figure 7 *In vitro* **neuronal differentiation of embryonic stem cells**
ES cells were pre-differentiated to neuronal progenitor cells via formation of embryoid bodies and subsequent suspension and adherent culture in appropriate differentiation medium. Figure modified from Doss *et al.*, 2004.

For preparation of embryoid bodies, stem cells were washed with PBS, trypsinised (trypsin/ EDTA) and resuspended in DMEM 10%. Using a multichannel pipette 20 µL drops of stem cell suspension ($1x10^4$ cells/mL) were transferred to petri dishes. The dishes were incubated upside down for two days (Figure 7). Thereafter, newly formed embryoid bodies were resuspended in 10 mL Iscove's 20%. After two more days in suspension culture, embryoid bodies were used in co-culture experiments or were further differentiated to neuronal precursor cells.

B1 Cell Culture

Two-hundred µL embryoid body suspension was seeded in gelatine-coated cell culture flask containing Iscove's 20% (Figure 7). The next day medium was exchanged to B1. Additionally, 12 µL EGF/ bFGF were added to culture medium on a daily basis. After eight days cells were transferred to B2 cell culture.

B2 Cell Culture

B1 cells were washed in PBS, treated with trypsin w/o EDTA, resuspended in B1 medium and redistributed into poly-L-lysine/ laminin coated cell culture flasks. Again, 12 µL EGF/ bFGF were added to the culture medium. The next day medium was exchanged to B2. Additionally, 12 µL EGF/ bFGF were added to culture medium daily.

2.5.4 Isolation and Culture of Primary Rat Brain Endothelial Cells

Primary rat brain endothelial cells were cultured from brain microvessels isolated from two-week-old Wistar rats based on the method by Szabo *et al*., 1997.

Experiments on animals were approved by the local government authorities of North-Rhine Westphalia. Rats were acquired from the institute's animal house and cared for in accordance with animal protection guidelines. Centrifugation steps were carried out in a Heraeus® Multifuge 3 S-R equipped with a swinging bucket rotor unless stated otherwise.

Ten to 12 animals, one at a time, were anesthetised with diethyl ether and sacrificed by decapitation. The skull was opened by sagital incisions and the brain was removed and placed in 25 mL PBS on ice. The cerebellum was cut off with a scalpel and discarded. The cerebrum was divided into the two hemispheres and the choroid plexus and white matter were removed. Using forceps the meninges were removed by spreading the hemispheres on sterile chromatography paper (Whatman cellulose filter paper, VWR International GmbH, Darmstadt, DE) and gentle rolling. Tissue pieces were then put in 1 mL DMEM on ice. All brains were processed in such a way. Then, the medium was aspirated and the tissue pieces were minced with forceps. By adding 13.5 mL DMEM, tissue pieces were transferred to a 50 mL Falcon tube. One mL collagenase (10 mg/mL) and 200 µL DNase (2,000 units/mL) were added and the tissue suspension was homogenised thoroughly with a glass pipette. The homogenate was then incubated on a rotating shaker (200 rpm) at 37°C for 45 min. After that, 10 mL DMEM were added to the mixture and centrifugated for 8 min at 1,000 x *g* (4°C). Then, the supernatant was aspirated; the cell pellet was thoroughly resupended in 25 mL filter-sterilised 20% BSA solution (in DMEM) (pH 7.4) and re-centrifugated for another 20 min at 1,000 x *g* (4°C). Then, the myelin layer that had formed and the BSA solution were aspirated. The remaining microvessel pellet was resuspended in 9 mL DMEM + 1 mL collagenase/ dispase (10 mg/mL) and 100 µL DNase (2,000 units/mL) and incubated on a shaker (200 rpm) at 37°C for 60 min. Thereafter, 10 mL DMEM were added to the cell suspension, and the suspension was centrifugated for 6 min at 700 x *g* (4°C). The supernatant was aspirated and 2 ml DMEM were added to the cell pellet. The pellet was gently homogenised and carefully placed on top of a 33% Percoll gradient. The gradient was centrifugated for 10 min at 1,000 x *g* (4°C). Using a long needle and syringe the interface between the formed red and white colour layers was collected and put into a new 50 mL Falcon tube containing 25 mL DMEM. The suspension was centrifugated for 10 min at 1,000 x *g* (4°C). Then, the supernatant was aspirated and the pellet resuspended in 15 mL DMEM and again centrifugated for 8 min at 700 x *g* (4°C). After aspiration of the supernatant, the digested isolated microvessel fraction remained. The microvessel pellet was resuspended in an appropriate amount of pRBEC medium and seeded onto fibronectin/ collagen IV-coated petri dishes. Over a period of 7 days *in vitro*, endothelial cells developed a continuous monolayer, which was used for experiments. In addition, cultures were regularly tested by immunocytochemistry and flow cytometry for factor VIII–related antigen (von Willebrand Factor, vWF) positivity.

2.6 Lateral Fluid Percussion Brain Injury and Brain Tissue Extract Production

All animal experimental procedures were carried out in accordance with animal protection guidelines and were approved by the local government authorities of North-Rhine Westphalia.

Male Sprague-Dawley rats were subjected to lateral fluid percussion (LFP) traumatic brain injury as described earlier (Dixon *et al.*, 1987; McIntosh *et al.*, 1989). In brief, the animals were anaesthetised with 60 mg/kg body weight pentobarbital intraperitoneally. Animals were placed in a stereotaxic frame and an opening, 5.0 mm in diameter, was made over the left parietal cortex. The fluid percussion device (Figure 8) was attached via a male Leur-loc which coupled with a female Leur-loc implanted over the exposed dura of the rat. Fluid percussion injury of moderate severity (2.4 atm) was induced as previously described (McIntosh *et al.*, 1989). Animals were sacrificed 45 min after brain injury. For the production of normal brain tissue extract, animals underwent craniotomy, but received no injury.

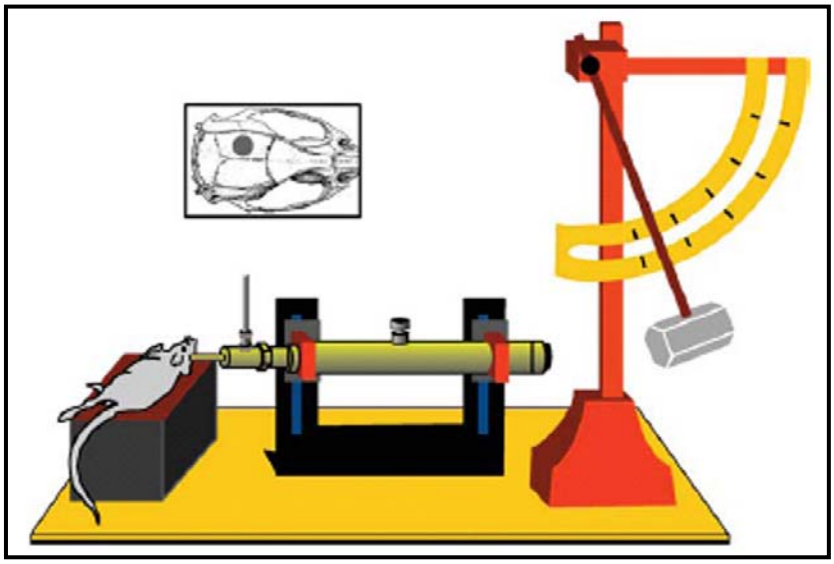


Figure 8 Schematic drawing of a lateral fluid percussion brain injury device

A pendulum from a known height impacts the piston of a saline-filled reservoir, forcing a brief fluid bolus into the sealed cranial cavity. This way a reproducible head injury is induced, simulating the clinical syndrome of a combined focal and diffuse brain injury. Picture from Thompson *et al.*, 2005.

Cerebral tissue extracts were derived by pooling and homogenising the respective cerebral hemispheres of traumatised rats as described by Chen and colleagues (Chen *et al.*, 2002a). Normal brain tissue extract controls were obtained from rats that had not received injury immediately following craniotomy. Briefly, left hemispheres of both experimental rats and control rats were placed on ice, and the wet weight in grams was rapidly measured. Subsequently, DMEM was added (150 mg tissue/ml DMEM) and tissue pieces were homogenised using an ultra sound homogeniser. After incubation on ice for 10 min, the homogenate was centrifugated for 10 min at 10,000 x *g* (4ºC). The supernatant was collected and stored at - 80ºC. Just before cell treatment, the homogenate was diluted 1:5 in serum-free DMEM/ Ham's F12.

2.7 Stem Cell Co-Culture with Endothelial Cells and Astrocytes

Contact Culture (Figure 9B)

Transwell™ filter inserts (pore size 0.4 µm, diameter 1.1 cm) were coated with fibronectin/ collagen IV on both sides. Endothelial cells (SV-ARBEC, pRBEC) or astroglia (C6) were plated on the underside of the membrane in 200 µl of medium at a concentration of $5x10^4$ cells/mL. The cells were left to adhere overnight. The next day dissociated embryoid bodies ($1x10^4$ cells) were seeded on top of the membrane.

Non-Contact Culture (Figure 9C)

Dissociated embryoid bodies ($1x10^4$ cells) were grown on top of fibronectin/ collagen IV coated filter inserts, while endothelial cells (SV-ARBEC, pRBEC) or astroglia (C6) were seeded on the fibronectin/ collagen IV coated bottom of the well.

Conditioned Medium (Figure 9A)

Dissociated embryoid bodies ($1x10^4$ cells) were grown on top of fibronectin/ collagen IV coated filter inserts in conditioned medium. Conditioned medium was derived from SV-ARBEC, pRBEC and C6, respectively.

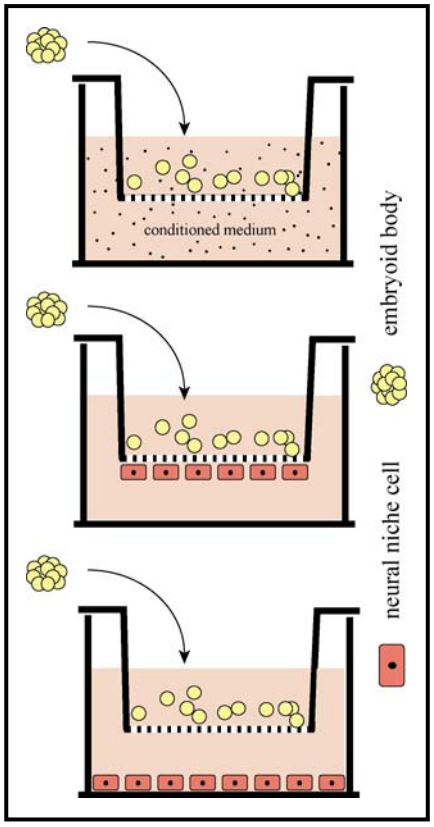


Figure 9 Co-culture settings for endothelial/ glial cell - stem cell interactions

Dissociated embryoid bodies (BAC7 embryonic stem cells) were cultivated in endothelial/ glial cell conditioned medium (A), contact (B) or non-contact co-cultures (C) with endothelial cells or astrocytes, respectively. A semi-permeable membrane separated cellular compartments.

Dissociated embryoid bodies ($1x10^4$ cells) attached to the top of fibronectin/ collagen IV coated filter insert and grown in standard medium in the absence of any additional cell types or conditioned medium were used as controls. All co-cultures were cultivated in serum-free DMEM/ Ham's F12 + antibiotics. Culture medium above and below the insert was carefully replaced every 48 hours and co-cultures were kept in a humidified atmosphere at 37°C for 7 days. Following co-culture, stem cells were harvested and total RNA was extracted.

2.8 Conditioning of ES Cell Culture with Brain Tissue Extract

For conditioning experiments stem cells (BAC7 and CGR8) were seeded at $1x10^5$ cells/ well in gelatine-coated 6-well plates. After a 24-hour attachment period, cells were incubated with serum-free DMEM/ Ham's F12 containing 20% cerebral tissue extract supernatant for up to 10 days. Treatment with serum-free DMEM/ Ham's F12 without any additives was considered as control. Medium was replaced after 3, 5, 7 and 10 days. Aspirated cell culture supernatant was collected, aliquoted and stored at - 80°C. Stem cells were harvested after respective time periods and total RNA was extracted. Additionally, CGR8 stem cells were subjected to proliferation assays at 3, 5 and 7 days of tissue extract treatment.

2.9 Molecular Biological Methods

2.9.1 Isolation of Total RNA

Total RNA was prepared from co-cultured embryonic stem cells using Qiagen's RNeasy Mini Kit following manufacturer's instructions. All centrifugation steps were carried out in a Heraeus® Biofuge pico unless mentioned otherwise. In the case of stem cell co-culture with endothelial or glial cells, stem cells were trypsinised and collected as a cell pellet prior to lysis to avoid any endothelial or astroglial cell contamination. This step was omitted in stem cell cultures conditioned by brain tissue extract. Here, stem cells were lysed directly in the culture vessel.

In general, cell culture medium was aspirated completely and cells were disrupted by the addition of 350 µL of Buffer RLT and vortexing. In order to homogenise the sample, the cell lysate was pipetted directly onto a QIAshredder spin column, placed in a 2 mL collection tube and centrifugated for 2 min at maximum speed. After that, 350 µL of 70% ethanol were added to the homogenised lysate and mixed well by pipetting. The sample was then applied to an RNeasy mini column placed in a 2 mL collection tube and centrifugated for 15 s at ≥ 8,000 x g. The flow-through was discarded. For on-column DNase digestion 350 µL Buffer RW1 were pipetted into the RNeasy mini column and centrifugated for 15 s at ≥ 8,000 x *g* to wash the sample. The flow-through was discarded. Ten µL DNase I stock solution were added to 70 µL Buffer RDD and mixed gently. The DNase I incubation mix was then added directly onto the RNeasy silica-gel membrane and placed on the benchtop for 15 min at RT. After the incubation time 350 µL Buffer RW1 were pipetted into the RNeasy spin column and centrifugated for 15 s at ≥ 8,000 x *g*. The flow-through was discarded and the RNeasy column was transferred into a new 2 mL collection tube. Five-hundred µL Buffer RPE were pipetted onto the RNeasy column and centrifugated for 15 s at ≥ 8,000 x *g* to wash the column. The flow-through was discarded. Again, 500 µL Buffer RPE were added to the RNeasy column and centrifugated for 2 min at ≥

8,000 x *g* to dry the RNeasy silica-gel membrane. Then, the RNeasy column was placed in a new 2 mL collection tube and centrifugated at full speed for 1 min. The RNeasy column was transferred to a new 1.5 mL collection tube and 50 µL RNase-free water were pipetted directly onto the RNeasy silica-gel membrane. The tube was centrifugated for 1 min at ≥ 8,000 x *g* to elute the RNA. Then, the tube was placed on ice and the RNA content and quality was determined by photometric analysis at 260 nm and 280 nm in an Ultrospec Plus Spectrophotometer. After that, RNA was aliquoted and stored at - 80°C.

2.9.2 Gel Electrophoresis

For determination of RNA quality, 1.2% FA gels were prepared and equilibrated in 1x FA gel running buffer for at least 30 min before RNA samples were loaded. RNA samples were prepared by combining 250 ng RNA, 5 µL RNA loading buffer (5x) and RNase-free water to 25 µL total volume. The mixture was incubated at 65°C for 5 min, chilled on ice and loaded onto the FA gel. Then, the gel was run at 60 V for 60 min in 1x FA gel running buffer.

PCR products were separated on 2% normal agarose gels (Sambrook *et al.*, 1989), running at 90 V for 30 min. 2 µL ethidium bromide (0.5 µg/mL) were added to 10 µL PCR product before it was loaded onto the gel.

After electrophoresis, RNA and DNA bands, respectively, were visualised on an UV-transilluminator TFX 20M with attached camera. Gels were photographed and signals of PCR products were semi-quantified using Gel-Pro® Analyzer Imaging Software.

2.9.3 Polymerase Chain Reaction

Prior to RT-PCR and cDNA synthesis, RNA was quantified spectrophotometrically, and its quality was determined by formaldehyde agarose gel electrophoresis and ethidium bromide staining. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for RT-PCR and cDNA synthesis, respectively. In all (RT-)PCR reactions negative controls were performed, substituting RNA by an equivalent amount of ddH_2O and expression of the house-keeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh)* was used as internal control. Semi-quantitative (RT-)PCR amplifications and cDNA syntheses were conducted in a Tpersonal thermal cycler and all sequence-specific primers used in these reactions (Table 2) were purchased from Carl Roth GmbH & Co, Karlsruhe, DE.

Semi-Quantitative Reverse Transcriptase PCR (RT-PCR)

Reverse transcription and polymerase chain reaction amplification was performed from 20 ng template RNA using SuperScript™ One Step RT-PCR System according to manufacturer's instructions. In brief, each RT-PCR reaction consisted of

25 µL Reaction Mix (2x)
2 µL template RNA
1 µL sense primer
1 µL antisense primer
1 µL RT/ Platinum® *Taq* Mix
20 µL ddH_2O

in a total volume of 50 µL. RT-PCR was performed with gene specific-primers according to the following cycling conditions:

cDNA synthesis and pre-denaturation:	45 min at 54°C	
	2 min at 94°C	
PCR amplification:denaturation:	15 s at 94°C	25 - 42 cycles
annealing:	30 s at respective temperature	
extension:	1 min at 72°C	
final extension:	10 min at 72°C	

cDNA Synthesis and Amplification

cDNA amplification was performed prior to PCR and qRT-PCR using the Advantage™ RT-for-PCR Kit according to manufacturer's instructions. In brief, total RNA (1 µg) was reverse transcribed in a total volume of 20 µL containing

1x reaction buffer
1 U/µL of RNase inhibitor
20 pmol random hexamer primer
0.5 mM of each deoxynucleotide
200 U of Moloney virus reverse transcriptase

The reaction was performed for 60 min at 42°C and for 5 min at 94°C to stop cDNA synthesis reaction and to destroy any DNase activity. Prior to reverse transcription, all RNA samples were denatured for 5 min at 70°C and cooled immediately on ice for 5 min to prevent secondary structure from reforming. The cDNA was stored at - 70°C until use.

Semi-Quantitative PCR

For PCR analysis 1 µL of cDNA was included in a total reaction volume of 50 µL. The PCR reaction mixture contained

1x PCR buffer
0.2 mM dNTP mix
sense and antisense primers, 0.1 µM each
1 unit of Platinum® *Taq* DNA Polymerase

Cycling conditions used for PCR amplification of cDNA consisted of

Initial step:	5 min at 94°C	
PCR amplification: denaturation:	30 sec at 94°C	25 - 42 cycles
annealing:	1 min at respective temperature	
extension:	1 min at 72°C	
final extension:	10 min at 72°C	

Quantitative Real Time PCR (qRT-PCR)

For all qRT-PCR reactions, equal amounts of input cDNA were used and studies were conducted in triplicates. For a single 20 µL reaction the following components were prepared:

10 µL *Taq*Man® Universal PCR Master Mix (2x)
1µL Assay-on-Demand™ gene expression assay mix
1 µL cDNA
8 µL RNase-free water

Amplification and detection were performed on 96-well plates using an ABI Prism 7700 Sequence Detector System with the following temperature profile:

Initial step:		2 min at 50°C	
PCR amplification:	denaturation:	10 min at 95°C	
	annealing:	15 s at 95°C	} 40 cycles
	extension:	1 min at 60°C	

Pre-designed *Taq*Man® Assay-on-Demand™ gene expression assays consisting of unlabled PCR primers and *Taq*Man® MGB probe (FAM™ dye-labeled) for *Gapdh* (NM_008084*), *POU5F1* (*Oct-4*) (NM_013633*), *nestin* (NM_016701*) and *microtubule-associated protein 2* (*MAP2*) (NM_008632*) were obtained from Applied Biosystems, Darmstadt, DE.

* National Center for Biotechnology Information (NCBI) accession number

Table 2 Primer sequences used for semi-quantitative (RT-)PCR and cDNA synthesis

Name (length)	Primer sequence	Temp./ Cycles
Fgf5 (465 bp)	sense 5'-AAA GTC AAT GGC TCC CAC GAA-3' antisense 5'-CTT CAG TCT GTA CTT CAC TGG-3' (NM_010203*)	58°C/ 42
Gapdh (183 bp)	sense 5'-AGA ACA TCA TCC CTG CAT CC-3' antisense 5'- CCT GCT TCA CCA CCT TCT TG-3' (NM_199472.1*)	54°C/ 25
Gata6 (574 bp)	sense 5'-GCA ATG CAT GCG GTC TCT AC-3' antisense 5'-CTC TTG GTA GCA CCA GCT CA-3' (NM_010258*)	58°C/ 37
Nestin (434 bp)	sense 5'-CTC GGG AGA GTC GCT TAG AG-3' antisense 5'-ATT AGG CAA GGG GGA AGA GA-3' (NM_016701*)	58°C/ 42
Neurofilament (625 bp)	sense 5'-TGA GCT GAG AAG CAC GAA GA-3' antisense 5'-TTG GTT GGT GAT GAG GTT GA-3' (NM_010910*)	54°C/ 40
Oct-4 (593 bp)	sense 5'-AGA GGG AAC CTC CTC TGA GC-3' antisense 5'-CTG GGA AAG GTG TCC CTG TA-3' (X52437*)	54°C/ 33
T brachyury (947 bp)	sense 5'-TGC TGC CTG TGA GTC ATA AC-3' antisense 5'-CCA GGT GCT ATA TAT TGC CT-3' (NM_009309*)	58°C/ 40

* National Center for Biotechnology Information (NCBI) accession number

2.10 Immunocytochemistry

All incubation steps were carried out at RT unless stated otherwise. For fixation cell culture medium was aspirated, cells were washed with PBS and incubated with either ice-cold methanol for 10 min or with FixA for 60 min depending on antibody. Cells were washed 4x in TBS and permeabilised in 0.55 M ammonium chloride/ 0.25% Triton (in TBS) for 10 min. Following 2 washes in TBS, unspecific binding sites were blocked by addition of 5% BSA in TBS for 60 min. Staining with primary antibody (Table 3) was carried out overnight at 4°C in 0.8% BSA (in TBS). The next day cells were washed 4x with TBS and blocked with 3% normal goat serum (NGS) and 0.8% BSA in TBS. Incubation with secondary antibody (Table 3) was carried out for 60 min in 3% NGS/ 0.8% BSA (in TBS). When biotinylated secondary antibodies were used, incubation was followed by 3 washes in TBS, and treatment with ABC Kit (according to manufacturer's instructions) for 20 min. After 2 more washes in TBS, cells were incubated with 3, 3'-diaminobenzidine (DAB) solution following manufacturer's protocol for 2-3 min. (These steps were omitted when fluorescent secondary antibodies were used. Following fluorescent secondary antibody incubation, it was directly proceeded with the following procedures.) Finally, cells were washed twice in TBS and soaked in ddH_2O for 5 min. After immunostaining slides were prepared with UltraMount and optionally, cover slips. Immunocytochemical stainings were analysed using either a Nikon Eclipse TS100 inverted microscope (CFI Achromat ADL10x/0.25 Ph1; CFI Achromat LWD ADL20xXF/0.4 Ph1; CFI Achromat LWD ADL40XF/0.55 Ph1) or a Nikon Eclipse E400 fluorescence microscope (FITC/GFP filter set: EX 465-495, DM 505, BA 515-555; PlanFluor 10x/0.3 Ph1, PlanFluor 20x/0.5 Ph1, PlanFluor 40x/0.75 Ph2). Controls for endothelial-specific antigen included pancreatic cell line ASPC-1 (negative) and endothelial cell lines b.End5 and HUVEC-c (positive). Immunostaining for neuronal antigens involved Neuro-2a and PC-12 cell lines as positive and endothelial cell line MHEC-5T as negative controls.

2.11 Fluorescence-Activated Cell Sorting

All reagents and consumables required for sample preparation, such as BD FACS™ Clean Solution, BD FACS™ Rinse Solution, BD FACS™ Lysing Solution, BD FACSFlow™ Sheath Fluid, BD Cytofix/ Cytoperm™ buffer, BD Cytofix™ buffer, BD Perm/ Wash™ buffer and BD CellWASH™ were purchased from BD Biosciences, Heidelberg, DE. Flow cytometric measurements were conducted in a FACScan, equipped with an air-cooled 488 nm argon-ion-laser (15 mW) with appropriate detector and filter settings (FITC = 530 nm; PE = 585 nm; PerCP > 650 nm) according to manufacturer's instructions. All centrifugation steps in FACS

(fluorescence-activated cell sorting) protocols were carried out for 5 min at 300 x *g* in a Heraeus® Multifuge 3 S-R with swinging bucket rotor.

2.11.1 Cell Proliferation Assay

The proliferation activity of stem cells was analysed by the rate of DNA synthesis using a BrdU-based flow cytometry kit according to manufacturer's instructions. In brief, 24 hours prior to flow cytometric analysis the appropriate cells were exposed to 5-bromo-2′-deoxyuridine (BrdU) by adding 10 µL BrdU solution (1 mM BrdU in 1x DPBS) directly to cell culture medium. The next day cells were detached from culture vessels by accutase II treatment, washed in PBS, pelleted by centrifugation and resuspended in BD Cytofix/ Cytoperm™ Buffer for fixation and permeabilisation. After 5 min incubation, the cells were washed with 1x BD Perm/ Wash™ Buffer and centrifugated. To expose the incorporated BrdU, cells were resuspended in 1x DPBS containing 300 µg/mL DNase and incubated for 60 min at 37°C. Afterwards, cells were washed in 1x BD Perm/ Wash™ Buffer, pelleted and resuspended in 50 µL diluted FITC-conjugated anti-BrdU antibody. Following incubation for 20 min at RT, the cells were washed with 1x BD Perm/ Wash™ Buffer, pelleted and finally resuspended in 0.5 mL staining buffer for flow cytometric analysis; elevated BrdU staining was associated with actively cycling as opposed to non-cycling cells.

2.11.2 Detection of Cell Type-Specific Antigens

For the detection of extracellular and intracellular cell-specific antigens, culture medium was aspirated from cell cultures, cells were detached from culture vessels by accutase II treatment, washed in PBS and pelleted by centrifugation (5 min at 300 x *g*). For detection of extracellular antigens, cells were resuspended in 5% FCS/ PBS (w Ca^{2+} + Mg^{2+}). Aliquots of 100 µL cell suspension were stained with appropriate primary antibody (Table 3) for 45 min at 37°C in the dark. Then, cells were washed with BD CellWASH™, spinned down and resuspended in 5% FCS (in PBS w Ca^{2+} + Mg^{2+}) containing the diluted secondary antibody (Table 3). Secondary antibody staining was carried out for 30 min at RT in the dark. Thereafter, cells were washed again with BD CellWASH™, pelleted and resuspended in 500µL 5% FCS (in PBS w Ca^{2+} + Mg^{2+}), and analysed in a FACScan. For intracellular antigen staining, cells were resuspended in BD Cytofix/ Cytoperm™ Buffer after centrifugation. After 5 min incubation, the cells were washed with 1x BD Perm/ Wash™ Buffer and re-centrifugated. Cells were again resuspended in 1x BD Perm/ Wash™ Buffer and stained with appropriate primary antibody (Table 3) for 60 min at 37°C. Thereafter, cells were washed in 1x BD Perm/ Wash™ Buffer, pelleted and resuspended in 1x BD Perm/ Wash™ Buffer containing an appropriate amount of fluorescent-labelled

secondary antibody (Table 3). Following incubation for 20 min at RT, cells were washed 1x BD Perm/ Wash™ Buffer, pelleted and resuspended in PBS for FACS analysis. Endothelial-specific antigen expression was controlled by FACS analysis of pancreatic cell line ASPC-1 (negative) and endothelial cell line HUVEC-c (positive). For determination of glial differentiation by A2B5-staining, undifferentiated embryonic stem cells served as negative control.

2.12 Enzyme-Linked Immunosorbend Assay

Kits for enzyme-linked immunosorbend assays (ELISA) for brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) detection were used to measure the time-dependent secretion of respective growth factors by embryonic stem cells. Cell culture supernatant aspirated from stem cell cultures conditioned by brain extract was subjected to immunological detection of BNDF, NGF and NT-3. Culture supernatant of undifferentiated ES cells and culture supernatant of ES cells grown in serum-free medium devoid of cerebral tissue extract supplementation were used as controls. All reagents and working standards were prepared as directed by the manufacturer and antibody-based detection of respective proteins was performed following manufacturer's instructions. Optical density measurements were carried out in a microplate reader at respective nm.

2.13 Statistical Analysis

Two-tailed student's t-test was used to evaluate differences between experimental samples and their respective controls. All values are expressed as mean ± SEM and are derived from at least three independent experiments. Unless stated otherwise p-values < 0.05 were considered statistically significant. Single values obtained from PCR analyses were normalised to respective expression of the house-keeping gene *Gapdh* and subsequently averaged. ELISA measurements were also normalised to background levels before individual experiments were averaged. Data generated by flow cytometry was intially averaged and thereafter normalised to negative control/ background. In general, data are presented relative to respective measurements in undifferentiated stem cells unless stated otherwise.

Table 3 Primary and secondary antibodies used for immunocytochemistry and FACS

Primary antibodies

Specifity	Isotype	Reactivity	Host	Dilution	Reference
Anti-neuron cell surface antigen (A2B5)	IgM	mouse	mouse	1:50	Chemicon International Ltd., Chandlers Ford, UK
Anti-neuronal nuclei (NeuN)	IgG_1	mouse	mouse	1:10	Chemicon International Ltd., Chandlers Ford, UK
Anti-neuron specific enolase (NSE)	IgG	rat	rabbit	1:2,000	Polysciences Europe GmbH, Eppelheim, DE
Anti-von Willebrand Factor (vWF)	IgG	human, mammals	rabbit	1:600	Sigma-Aldrich Chemie GmbH, Munich, DE

Secondary antibodies

Specifity	Isotype	Reactivity	Host	Dilution	Reference
FITC-conjugated anti-rabbit immunoglobulins	$F(ab')_2$	rabbit	swine	1:10	DakoCytomation GmbH, Hamburg, DE
PerCP-Cy5.5-conjugated anti-mouse IgM	$IgG_{2a,\kappa}$	mouse	rat	1:100	BD Biosciences, Heidelberg, DE
Biotinylated anti-mouse immunoglobulins	IgG	mouse	goat	1:200	DakoCytomation GmbH, Hamburg, DE
Biotinylated anti-rabbit immunoglobulins	IgG	rabbit	goat	1:200	DakoCytomation GmbH, Hamburg, DE

3 RESULTS

3.1 Co-Culture Manipulates Cell Lineage Determination of ES Cells

Stem cell self-renewal and differentiation *in vivo* is thought to be guided and controlled by the cellular and molecular environment in which the stem cell resides. Elucidating the role of the microenvironmental impact on stem cell endogenous properties is of particular importance for clinical applications in CNS cell replacement therapies. Brain endothelial and glial cells contribute to the cerebral stem cell niches *in vivo* and have been proposed to control recruitment and maintenance of neural stem cells. The significance of endothelial cells and astrocytes for guiding embryonic stem cell fate has previously not been investigated, but might be essential for ES cell-based transplantation paradigms where neural stem cells are not applicable. Hence, in a first experimental approach this study examined the influence of cerebral microvascular endothelial cells and astrocytes on ES cell's differentiation capacity in an *in vitro* co-culture model. Therefore, primary microvascular endothelial cells were prepared from brains of neonatal rats by a standardised protocol (Szabo *et al.*, 1997). Initially, microvessel fragments were separated from brain tissue by gradient centrifugation and enzymatic digestion and seeded onto collagen/ fibronectin-coated cell culture dishes (Figure 10A). Already 1 day after isolation, endothelial cells started to migrate out from single microvessel fragments (Figure 10B). By day 7 of *in vitro* culture, endothelial cells had formed a continuous monolayer of non-overlapping, tightly packed cells. Primary rat brain endothelial cells (pRBEC) displayed so-called "fibroblast-like" morphology typical of cerebral microvascular cells: fusiform cell-shape with oval nucleus in the center and neighbouring cells tightly apposed to each other (Figure 10C). Relative purity of primary cell cultures was regarded crucial for subsequent experiments. Isolated pRBEC were analysed for the expression of endothelial-specific marker von Willebrand Factor (factor VIII-related antigen) upon confluence by both, immunocytochemistry and flow cytometry. Von Willebrand Factor antigen was typically localised in storage granules of the cytoplasm, distributed around the nucleus (Figure 10D, E). Additionally, fluorescent staining of primary endothelial cells impressively demonstrated the vessel forming character of this cell type (Figure 10E). FACS analysis demonstrated relative purity of primary cell culture; on average > 70% of cells from single pRBEC culture displayed positive staining for von Willebrand Factor and were thus identified as cerebral microvascular endothelial cells (Figure 10F). Minor contaminations possibly resulted from pericytes and fibroblasts, co-purified during pRBEC isolation procedure.

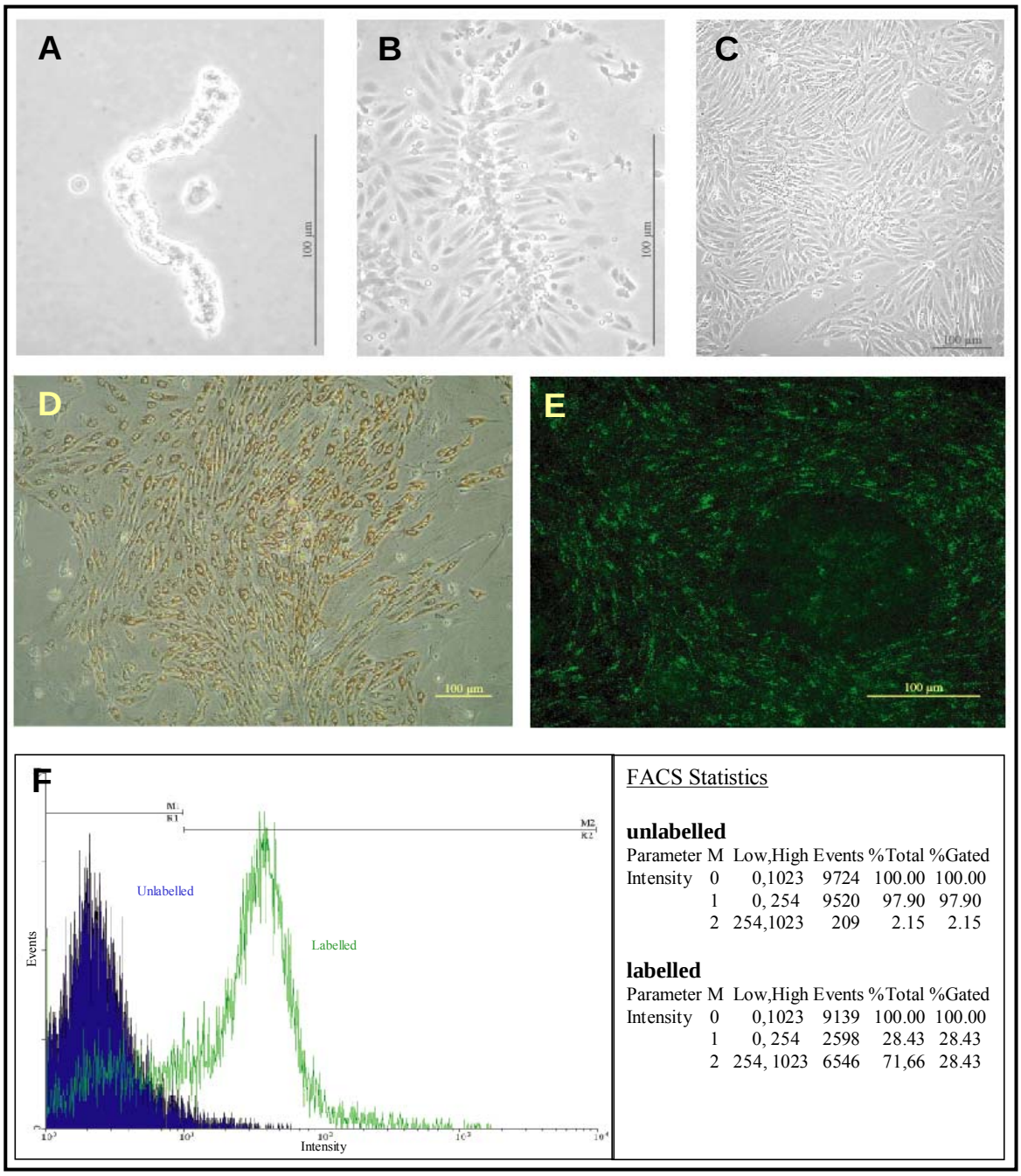


Figure 10 Isolation and identification of primary rat brain endothelial cells

Isolated microvessels were initially seeded onto collagen-coated culture dishes (A). After 24 hours incubation, endothelial cells started to migrate out of microvessels (B) and over a 7-day culture period, cerebral endothelial cells formed near to confluent monolayers (C). Monolayers were examined for von Willebrand Factor expression by immunocytochemical staining with biotinylated (D) and fluorescent secondary antibodies (E). FACS analysis of pRBEC monolayers with FITC-labelled von Willebrand Factor antibody yielded on average > 70% positive cells (F).

Photographs were taken using either a Nikon Coolpix 5000 mounted on a Nikon Eclipse TS100 inverted microscope or a Nikon Eclipse E400 fluorescence microsope with attached CCD camera. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

For co-culture experiments a rat brain endothelial cell line, immortalised by introduction of the SV40 viral T-antigen (SV-ARBEC) and a rat brain glioma cell line (C6) were chosen in addition. Then, cell culture inserts consisting of a semi-permeable membrane at their bottom were prepared for three different experimental settings as described in Chapter 2.7, Figure 9. For contact co-culture, BAC7 embryonic stem cells and respective endothelial or glial cells were cultivated on opposite sides of the same collagen-coated membrane, allowing cell-cell contacts to establish through membrane pores. For non-contact co-cultures BAC7 embryonic stem cells were grown on the top of the semi-permeable collagen-coated membrane, whereas endothelial cells or astrocytes were grown on the bottom of the corresponding well. For conditioned medium cultures, BAC7 embryonic stem cells were grown on collagen-coated culture inserts in conditioned medium derived of respective endothelial or glial cell types. After 7 days of co-culture, ES cells were harvested and total RNA was extracted. Thereafter, RNA obtained from co-cultured ES cells was examined for the expression of pluripotency marker *Oct-4*, neural progenitor marker *nestin* and early neuronal marker *neurofilament* (68 kDA) by semi-quantitative RT-PCR.

In these experiments, undifferentiated BAC7 embryonic stem cells and pre-differentiated neuronal precursor cells were used as differentiation controls. Pre-differentiation of BAC7 embryonic stem cells followed a previously published protocol by Arnhold *et al.*, 2000b. Via a three-step protocol (Chapter 2.5.3, Figure 7), ES cells were first aggregated into embryoid bodies, where ES cells reside as multicellular structures of compact round shape (Figure 11B).

Following aggregation embryoid bodies were kept in suspension culture before being plated onto differentiation-promoting substrates. In adherence culture single pre-neuronal cell started to migrate out of embryoid bodies (Figure 11C) and after 2 more weeks in differentiation medium, small neuronal cells had formed, characterised by polygonal morphology and small axonal outgrowths (Figure 11D). Total RNA was isolated from undifferentiated ES cells and pre-differentiated neuronal precursors, and analysed for the expression of developmental markers in the same way as from experimental cultures. In the following gene expression data is presented as amount relative to respective gene expression level found in undifferentiated ES cells.

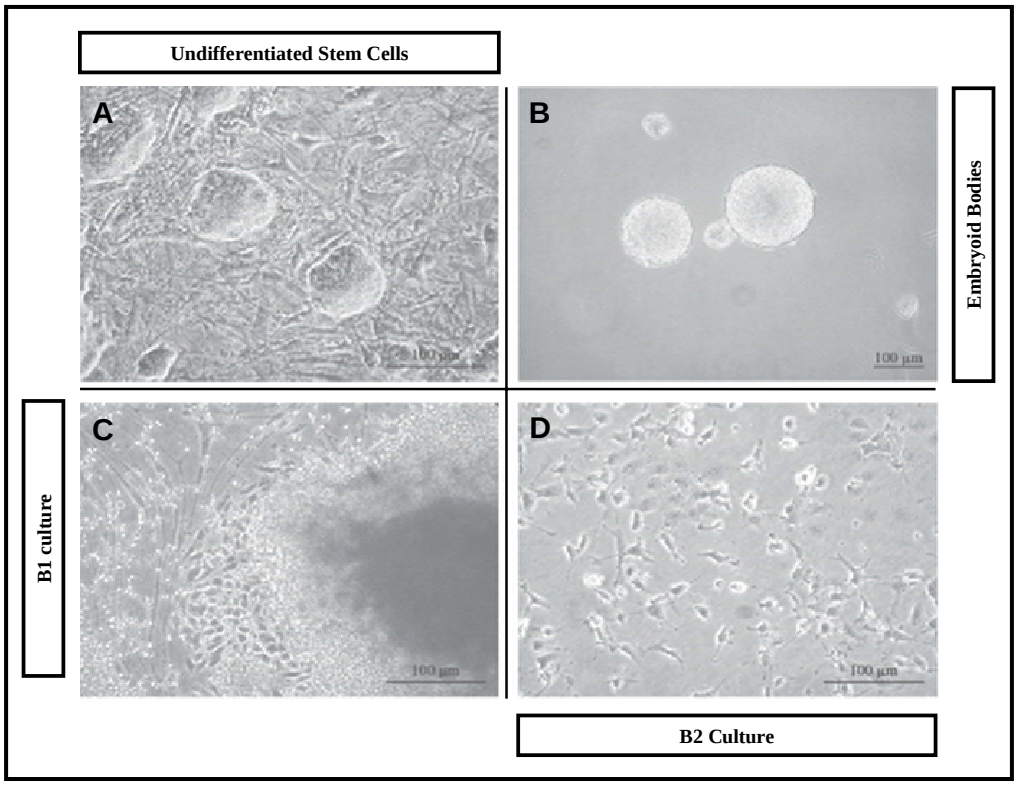


Figure 11 Pre-differentiation of BAC7 embryonic stem cells to neuronal precursor cells

Undifferentiated BAC7 embryonic stem cells were grown on MEF feeder layer (A). After aggregation of embryoid bodies, spheroids were cultivated in suspension culture (B). In B1 adherence cell culture immature neural cells started to separate from cellular aggregates (C) and during B2 differentiation culture cells flattened and developed into enriched population of small, flat cells with apolar, bipolar, or short multipolar processes (D). Photographs were taken with a Nikon Coolpix 5000 mounted on a Nikon Eclipse TS100 inverted microscope. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

3.1.1 Onset of Differentiation in ES Cells is Delayed in Co-Cultures

Upon *in vitro* differentiation of ES cells towards neuronal precursor cells (NP) a significant reduction of *Oct-4* mRNA levels (0.07 ± 0.04) could be detected in NP compared to undifferentiated stem cells (Figure 12A). In stem cells grown on collagen-coated cell culture inserts a minor decrease in *Oct-4* expression could also be observed (0.8 ± 0.3). This downregulation of pluripotency marker *Oct-4* was supportive for the onset of differentiation events in both cell types. Furthermore, significant downregulation of *Oct-4* in neuronal precursor cells indicated an advanced state of differentiation compared to ES cells grown on collagen. However, after contact or non-contact co-culture with C6 or SV-ARBEC *Oct-4* expression level of stem cells remained more or less unaltered, similar to *Oct-4* levels in undifferentiated ES cells (Figure 12A). This indicated the inhibition of differentiation events in stem cells by endothelial

or astroglial cells. Only in conditioned medium cultures, astroglia-conditioned medium caused slightly reduced *Oct-4* expression (0.8 ± 0.1) and endothelial-conditioned medium initiated increased *Oct-4* expression in stem cells (1.3 ± 0.6). This increase in *Oct-4* expression levels was also observed in all cultures, where stem cells were cultivated with freshly isolated primary endothelial cells of the rat brain (pRBEC) or with conditioned medium derived thereof (contact: 1.3 ± 0.2, non-contact: 1.2 ± 0.2, conditioned: 1.6 ± 0.2) (Figure 12A). Both, the down- and the upregulation of pluripotency marker *Oct-4* can be perceived as ongoing differentiation actions in stem cells (Niwa *et al.*, 2000; Shimozaki *et al.*, 2003). However, prolonged *Oct-4* expression in contact and non-contact cultures with astroglial or immortalised endothelial cells indicated suppression of differentiation onset in ES cells.

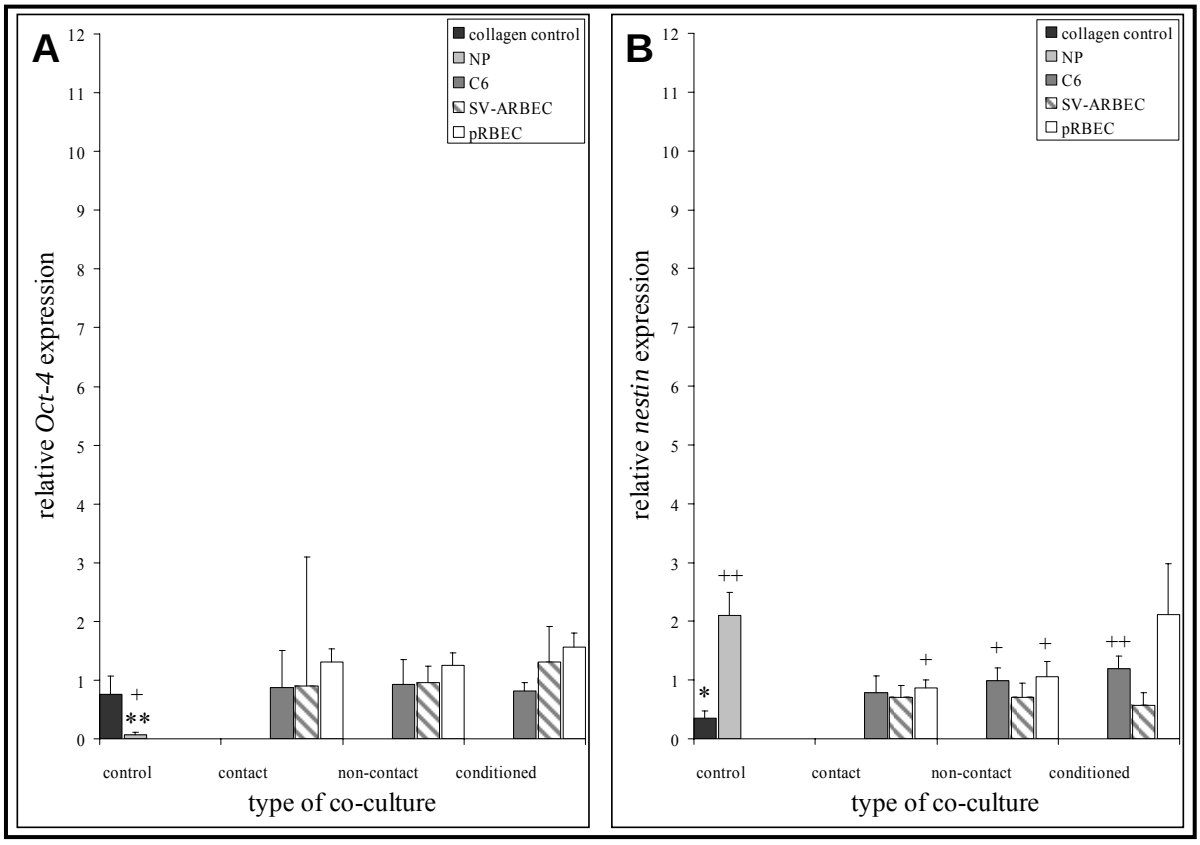


Figure 12 RT-PCR analysis of *Oct-4* and *nestin* expression in co-cultured ES cells

Developmental marker genes were analysed by RT-PCR and revealed the prolonged expression of pluripotency marker *Oct-4* in co-cultured stem cells, which was at the same time downregulated in neuronal precursor cells and in stem cell cultures alone (A). In addition, expression of neural progenitor cells marker *nestin* was restricted to undifferentiated stem cell levels in co-cultured stem cells (B); only stem cells cultivated in pRBEC-conditioned medium showed elevated *nestin* expression (B).

Data is presented as mean ± SEM and represents relative amounts of gene expression compared to undifferentiated BAC7 embryonic stem cells. Significance vs. undifferentiated cells: * = $p < 0.05$; ** = $p < 0.01$; significance vs. collagen control $^{+}$ = $p < 0.05$; $^{++}$ = $p < 0.01$. (collagen control: ES cells cultivated alone on collagen-coated culture inserts, NP: neuronal precursor cells, C6: glioma cells, SV-ARBEC: immortalised rat brain endothelial cells, pRBEC: primary rat brain endothelial cells)

3.1.2 Neuronal Lineage Commitment is Inhibited upon Co-Culture

Analysing neuronal differentiation status of pre-differentiated neuronal precursor cells, these cells demonstrated an increase of *nestin* mRNA levels up to 2.1 ± 0.4 compared to undifferentiated stem cells (Figure 12B). Expression of this neural progenitor cell marker indicated commitment of neuronal precursor cells to a neural fate. In contrast, stem cells grown on collagen-coated culture inserts displayed a significantly decreased *nestin* expression (0.4 ± 0.1) compared to undifferentiated stem cells (Figure 12B), either implying a more advanced state of neural lineage commitment by downregulation of *nestin* or supporting commitment to a other than neural cell fate. A downregulation of *nestin*, although by far not as strong as observed in cells grown on collagen, was detected in all stem cell co-cultures with SV-ARBEC (contact 0.7 ± 0.2, non-contact 0.7 ± 0.2, conditioned: 0.6 ± 0.2). In co-cultures with C6 glioma cells (contact 0.8 ± 0.3, non-contact 1.0 ± 0.2, conditioned: 1.2 ± 0.2) and in contact and non-contact culture with pRBEC (contact 0.9 ± 0.1, non-contact 1.1 ± 0.3) (Figure 12B), stem cells exhibited no significant alterations to *nestin* levels of undifferentiated stem cells. Indeed, expression of *nestin* in co-cultured stem cells was significantly restricted to undifferentiated stem cell levels compared to downregulated *nestin* levels in stem cells cultivated alone (Figure 12B). Thus, neural lineage commitment seemed to be restrained when stem cells were co-cultured with either endothelial or glioma cells. However, an induction of *nestin* expression was observed in stem cells cultivated in pRBEC-conditioned medium (2.1 ± 0.9), even exceeding *nestin* mRNA levels measured in neuronal precursor cells (Figure 12B). This observation strongly indicated that cells grown in endothelial cell-conditioned medium were committed to a neural cell fate.

Similar to *nestin* expression, a significant upregulation of early neuronal marker *neurofilament* was observed in pre-differentiated neuronal precursor cells (3.4 ± 0.4) (Figure 13A). Here, *neurofilament* expression more than trebled compared to levels measured in undifferentiated stem cells, supporting the hypothesis that in precursor cells neuronal structures were created, and thus, neuronal lineage commitment occurred. A 2-fold increase in *neurofilament* mRNA levels was also observed in stem cells grown on collagen (2.1 ± 0.9). *Neurofilament* expression in stem cells of all contact and non-contact co-cultures were also slightly elevated, but remained below levels measured in cell cultivated on collagen alone (Figure 13A). These observations suggested the suppression of neuronal differentiation events in stem cells by endothelial and glial cell co-culture. In contrast, significantly elevated *neurofilament* expression could be detected in stem cells grown in conditioned medium of all three cell types (SV-ARBEC: 2.8 ± 0.5, C6: 2.2 ± 0.2, pRBEC: 3.6 ± 0.8). Amounts measured

exceeded control levels and in the case of pRBEC even surpassed levels measured in neuronal precursor cells (Figure 13A), indicating that neuronal lineage commitment could be induced by endothelial cells and astrocytes, if induction was mediated via cell contact-independent cues.

Differentiation towards other than ectodermal lineages, especially the possibility of development of an endothelial phenotype was excluded, as demonstrated by lack of early endothelial marker *Flk-1* expression (data not shown).

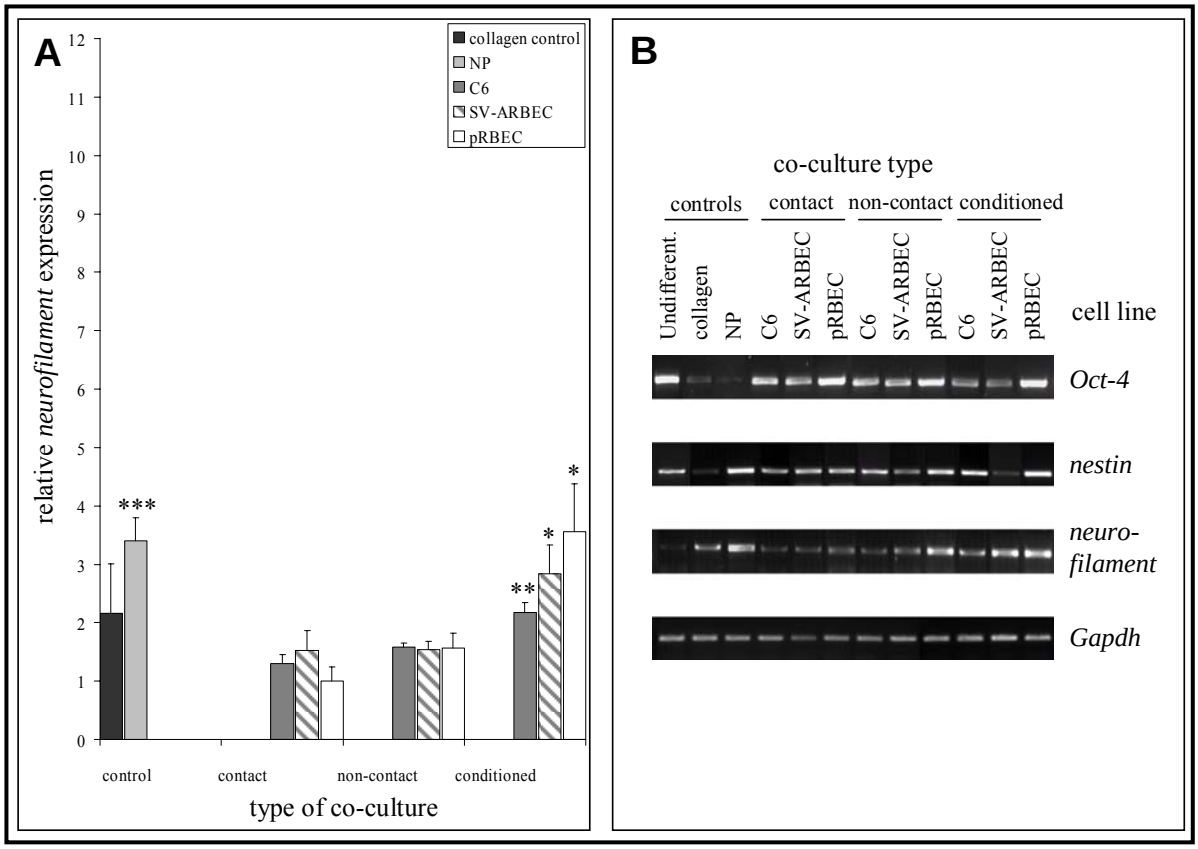


Figure 13 RT-PCR analysis of early neuronal marker *neurofilament* in co-cultured ES cells

Similar to *nestin*, expression of neuronal cell marker *neurofilament* was restrained in co-cultured stem cells, and only lightly elevated above undifferentiated cell levels in contact and non-contact co-cultures (A). However, when grown in endothelial or glial cell-conditioned medium, stem cells exhibited increased expression of *neurofilament*, corresponding to enhanced *neurofilament* levels in neuronal precursor cells and stem cells cultured alone (A). These results indicated that the presence of endothelial and glial cells of the neural stem cell niche generally inhibited neuronal differentiation of stem cells, but that humoral factors alone readily induced neuronal lineage commitment. In (B) representative gel electrophoresis of RT-PCR products of individual developmental markers are depicted.

Data is presented as mean ± SEM and represents relative amounts of gene expression compared to undifferentiated BAC7 embryonic stem cells. Significance vs. standard: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (undifferent.: undifferentiated BAC7 ES cells, collagen control: ES cells cultivated alone on collagen-coated culture inserts, NP: neuronal precursor cells, C6: glioma cells, SV-ARBEC: immortalised rat brain endothelial cells, pRBEC: primary rat brain endothelial cells)

3.1.3 Cellular Brain Microenvironment Induces Glial Differentiation in ES Cells

In addition to expression analysis for neuronal differentiation markers by RT-PCR, co-cultured stem cells were analysed for the expression of an oligodendroglial lineage marker by flow cytometry. A2B5 is an antigen found on the cell surface of glial progenitor cells and can be detected by FACS via binding of an A2B5-specific antibody and subsequent fluorescent labelling. Flow cytometric analysis detected similar percentages of A2B5-expressing cells in cultures of pre-differentiated neuronal precursor cells and in stem cell cultures on collagen-coated culture inserts. Both cultures displayed significantly increased numbers of A2B5-expressing cells compared to undifferentiated stem cell culture (NP: 1.7 ± 0.5; collagen control: 2 ± 2). Furthermore, the number of cells expressing A2B5 antigen was massively and significantly increased in all co-cultured stem cells, independent of co-culture setting and cell type co-cultured with (4- to 8-fold increase in A2B5-expressing cells compared to undifferentiated stem cells) (Figure 14). Numbers of A2B5-positive co-cultured stem cells exceeded amounts of A2B5-positive cells measured in control cell cultures by far, indicating the strong induction of oligodendroglial lineage commitment by co-culture. Compared to undifferentiated cells, strongest effects were observed in all co-cultures in which primary endothelial cells (pRBEC) were involved (contact 7 ± 3, non-contact 8 ± 3, conditioned: 8.253 ± 0.007), as well as upon culture in SV-ARBEC-conditioned medium (8 ± 1) (Figure 14). This suggested that endothelial cells rather than astrocytes themselves initiated stem cell commitment to a glial cell fate. Likewise the induction of neuronal differentiation, a greater inductive potential for glial lineage commitment must be assigned to the action of humoral factors than to cell contact-mediated influences.

3.1.4 Conditioned Medium Promotes Formation of Distinct Neuronal-Like Stuctures

To verify quantitative results obtained by RT-PCR and flow cytometry, stem cell co-cultures were examined by phase contrast and fluorescent microscopy. At the beginning of co-culturing, most stem cells resided within multicellular aggregates close to each other. Then, they started growing into sheets of largely flattened progeny, maintaining cell-cell contact. After only 5 days in co-culture, single cells detached from these spherical aggregates and formation of neuronal-like structures could be observed at the outskirts of cell spheroids. Especially stem cells that were grown in pRBEC- and SV-ARBEC-conditioned medium for the proposed period exhibited extensive neurite-like outgrowths, similar to axonal sprouting events observed in neurons (Figure 15). Moreover, processes of individual cells started to grow towards each other mimicking the establishment of synaptic connections (Figure 15F). Additionally, differences in size of cells and

in length and number of cellular outgrowths could be detected. While stem cells cultivated in SV-ARBEC-conditioned medium were rather small with short bipolar or more rarely, multipolar processes, stem cells grown in pRBEC-conditioned medium displayed complex arborised outgrowths and cells were generally bigger in size. These observations supported differential inductive potential of endothelial cells of different origin already discovered during gene expression analysis.

Furthermore, embryonic stem cell line BAC7 used in these experiments constitutively expressed the reporter protein GFP, facilitating identification of stem cells in co-culture. Using a fluorescent microscope, observed neuronal-like structures could clearly be associated with ES cells (Figure 16).

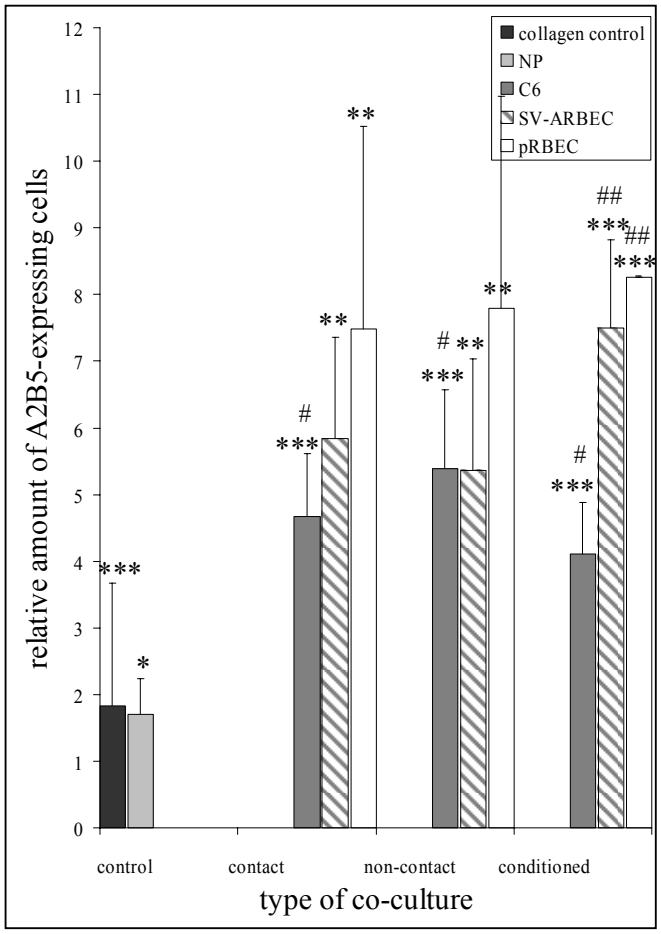


Figure 14 FACS analysis of A2B5 expression in co-cultured stem cells

FACS analysis showed elevated numbers of A2B5-expressing cells in neuronal precursor cell and in control stem cell culture. In ES cell co-cultures with endothelial and glial cells of the *in vivo* neural stem cell niche 3-times more A2B5-antigen presenting cells were detected than in undifferentiated stem cells. This indicated the induction of extensive oligodendroglial differentiation events in co-cultured stem cells. Data is presented as mean ± SEM and represents relative amounts of A2B5-positive cells compared to undifferentiated BAC7 embryonic stem cells culture. Significance vs. undifferentiated BAC7 stem cells: * = $p < 0.05$; ** = $p < 0.01$ *** = $p < 0.001$; Significance vs. neuronal precursors $^{\#} = p < 0.05$; $^{\#\#} = p < 0.01$. (collagen control: stem cells cultivated alone on collagen-coated culture inserts, NP: neuronal precursor cells, C6: glioma cells, SV-ARBEC: immortalized rat brain endothelial cells, pRBEC: primary rat brain endothelial cells)

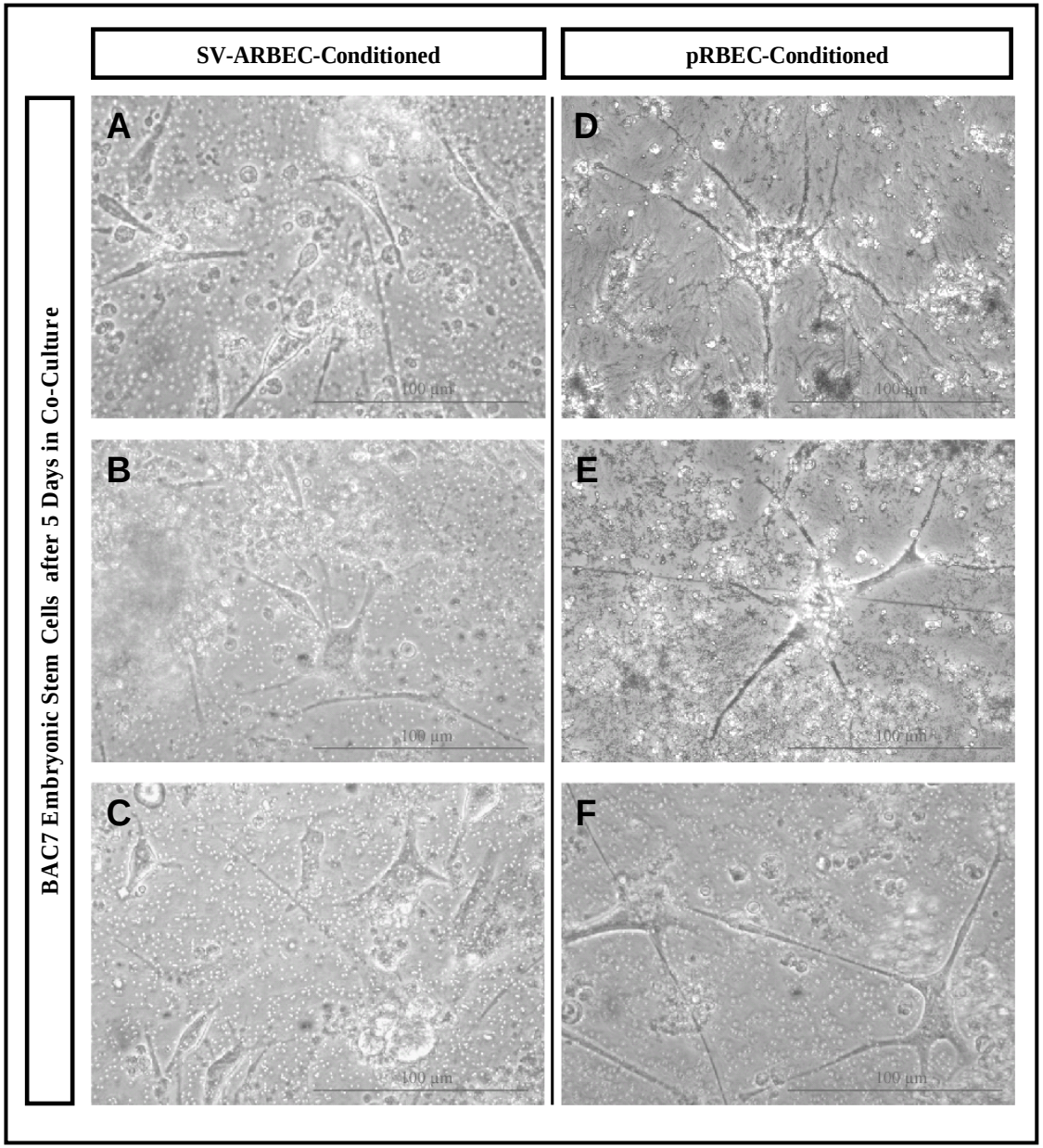


Figure 15 Phase contrast microscopy of ES cells in endothelial cell-conditioned medium

Culture of ES cells in conditioned medium derived either of primary cerebral endothelial cells (pRBEC) or of an immortalised brain endothelial cell line (SV-ARBEC) exhibited neuronal-like morphologies (A-F). After only 5 days of co-culture, single small cells that had separated from cellular aggregates flattened and grew bipolar or multipolar processes of varying length (A-C). Few cells appeared larger than others and displayed complex arborised processes (D, E). In addition, some cells seemed to develop synapse-like structures with neighbouring cells (F). Small reflecting circular structures in photographs are pores of the underlying semi-permeable membrane, which became also visible by phase contrast microscopy. Photographs were taken with a Nikon Coolpix 5000 mounted on a Nikon Eclipse TS100 inverted microscope. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

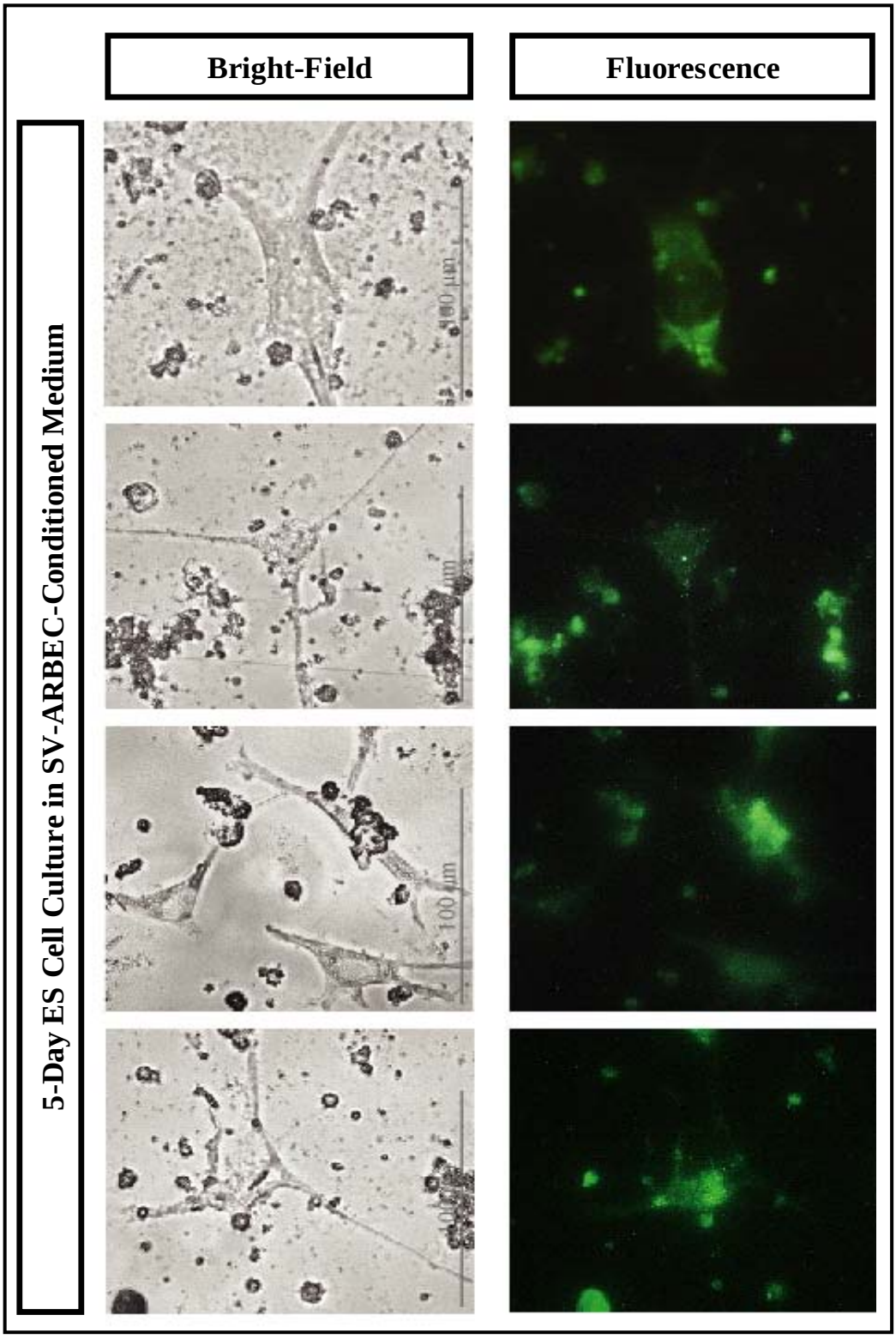


Figure 16 Fluorescence microscopy of GFP-expressing ES cells in conditioned medium

GFP-expression of BAC7 embryonic stem cells facilitated the association of observed neuronal-like morphologies with differentiating stem cells via fluorescence microscopy. Photographs show BAC7 embryonic stem cells cultured in SV-ARBEC-conditioned medium for 5 days. Individual stem cells display distinct neuronal-like structures, such as elongated multipolar processes originating from flat polygonal cellular bodies. Photographs were taken with a Nikon Eclipse E400 fluorescence microscope and attached CCD camera. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

3.2 Post-Traumatic Microenvironment Changes ES Cell's Gene Expression Profile

Traumatic brain injury is associated with a massive loss of multiple cell types due to primary insult and secondary sequelae. Cell transplantation strategies to restore the damaged nervous tissue have in the past primarily focused on the implantation of stem cells. Using a clinically validated lateral fluid percussion model for traumatic brain injury (Figure 8), our laboratory has previously investigated the effects of stem cell transplantation into the traumatised brain of rats. In a study by Riess and co-workers, BAC7 embryonic stem cells were transplanted into the cortex of male Wistar rats 72 hours post-lateral fluid percussion brain injury. Neurologic motor function was assessed in animals before injury and 24 hours, 1, 3 and 6 weeks after injury-induction and subsequent stem cell transplantation. It was shown that brain-injured animals that had received stem cell transplantation performed significantly better on the Rotarod and in Composite Neuroscore Tests (Figure 17) (Riess, unpublished data). Both experimental test settings represent standardised, well-established tests of balance, vestibulomotor function and coordination and have been shown to correlate with injury severity and to be sensitive to pharmacological interventions (McIntosh *et al.*, 1989; Riess *et al.*, 2001).

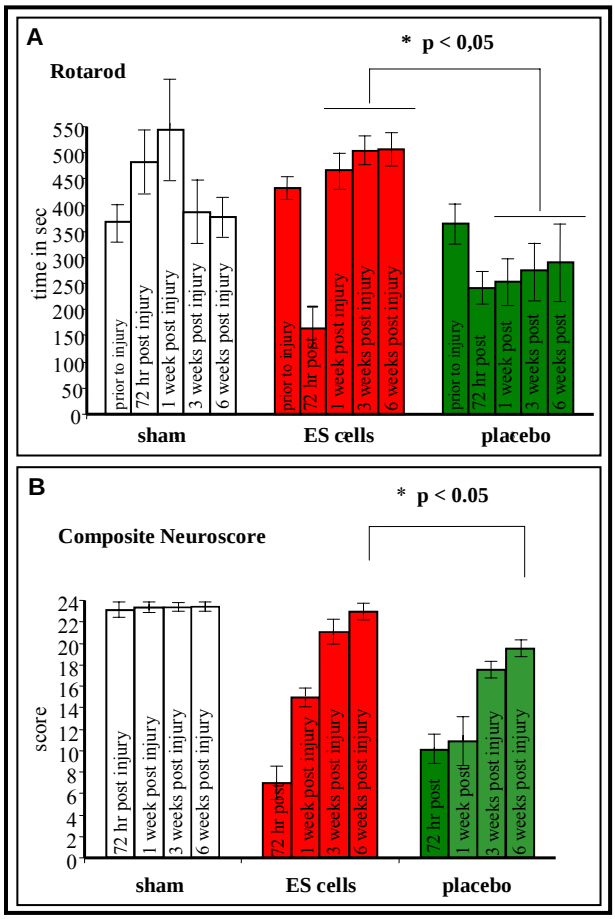


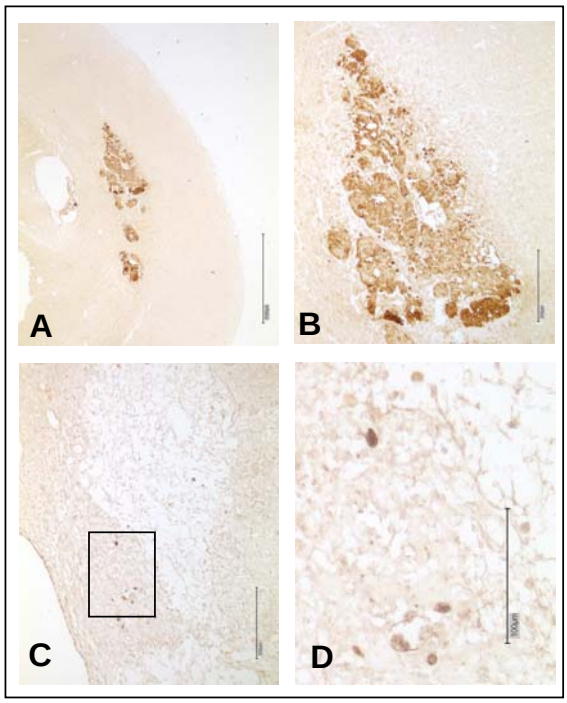
Figure 17 Rotarod and Composite Neuroscore results following ES cell transplantation

Purpose of the Rotarod test is to assess a rodent's sensorimotor coordination. Therefore, the animal is placed on a rotating rod, whose speed is gradually increasing. The rodent's ability to remain on the rotating rod is recorded. The Composite Neuroscore test protocol employs a previously described battery of neurological tests primarily concerned with fore- and hindlimb flexion (McIntosh *et al.*, 1989).
Prior to and following lateral fluid percussion brain injury with and without subsequent ES cell transplantation, animals were evaluated for behavioural deficits.
A Rotarod: brain-injured animals showed functional impairment 72 hours after injury. Within 1 week following ES cell transplantation, brain-injured animals performed significantly better on the rotating rod as injured placebo-injected animals.
B Composite Neuroscore: injured animals displayed significant impairment in motor function at 72 hours post-injury. However, 3 and 6 weeks post-transplantation injured animals showed significant improvement in their composite neuroscore in comparison to placebo-treated animals.
Data is presented with kind permission of Peter Riess (unpublished data).

Evaluating the cellular basis for functional improvements observed, Molcanyi and colleagues examined brain sections of animals sacrificed at day 5 or week 7 post-injury and post-stem cell transplantation by immunohistochemistry. Up to five days after implantation clusters of GFP–positive ES cells could be detected at the implantation site (Figure 18A, B). However, after 7 weeks only small amounts of ES cells were left in the brain tissue, indicating the extensive loss of implanted cells over the observed time (Figure 18C, D).

Figure 18 Coronal sections of injured rat brain following ES cell transplantation

Seventy-two hours post-lateral fluid percussion brain injury, animals received BAC7 embryonic stem cell transplantation ipsilaterally. 5 days post-implantation, anti-GFP immunostaining detected clusters of GFP-positive ES cells (brown) close to implantation site (A, B). 7 weeks post-transplantation, only few GFP-positive cells were left at former implantation site (C, D). Photographs are presented with kind permission of Marek Molcanyi (unpublished data).



Upon closer investigation, Molcanyi and colleagues demonstrated the extensive phagocytosis of implanted stem cells by activated microglia and macrophages (Figure 19) (Molcanyi, unpublished data). Thus, a significant effect of post-traumatic inflammatory response on stem cell survival and integration in the traumatically injured brain could be delineated. Yet, in view of diminishing numbers of grafted cells, the reason for significant neurological recovery remained unclear. In order to further elucidate the impact of the pathological environment caused by traumatic brain injury on proliferation and gene expression of embryonic stem cells, as well as to explain functional improvements observed, this study established an *in vitro* system, in which CGR8 embryonic stem cells were grown in the presence of brain tissue extract. CGR8 embryonic stem cells were chosen because they could be cultivated in feeder-free conditions, which facilitated analysis of exogenous influences on stem cells enormously. The cerebral tissue extract was either prepared from normal (uninjured) rat brains or from rat brains injured by lateral fluid percussion (Chapter 2.6).

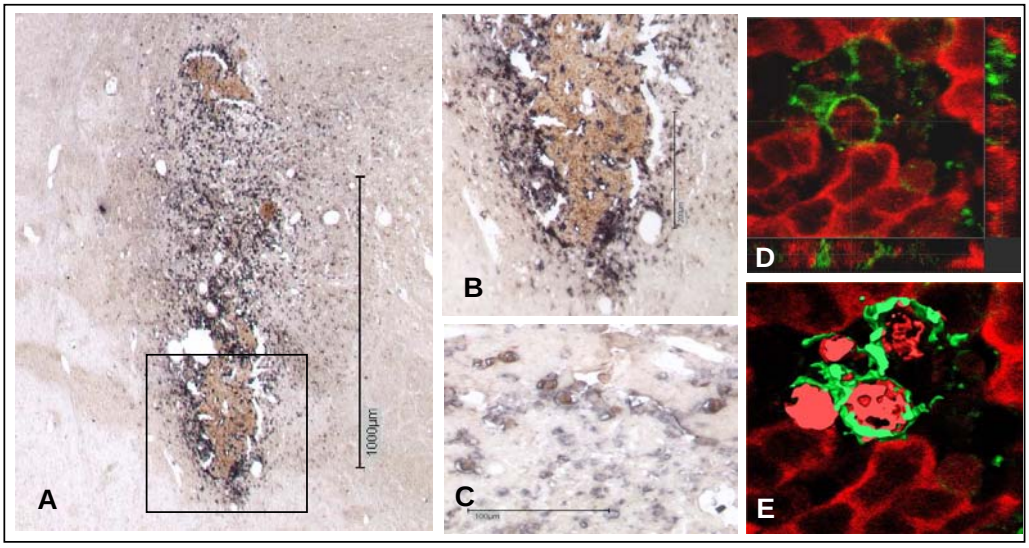


Figure 19 Phagocytosis of ES cells transplanted into traumatically injured rat brain

Following contralateral transplantation of BAC7 embryonic stem cells, numerous macrophages (blue) surrounding ES cell cluster (brown) were detected 5 days post-implantation by ED-1/ GFP double staining (A, B). Blue-stained macrophages incorporating individual brown-coloured ES cells were observed under higher magnification (C), indicating phagocytosis of implanted cells. Confocal laserscanning microscopy allowed topographical 3D reconstruction of phagocytosis process (D, E): macrophages (green) infiltrating stem cell cluster (red) (D); stem cell remnant (red) incorporated by phagocyting macrophage (green) (E). Pictures are reprinted with kind permission of Marek Molcanyi (unpublished data)

CGR8 embryonic stem cells were cultivated in serum-free culture medium supplemented with 20% brain tissue extract for up to 7 days. At different time points during the experiment (after 3, 5 and 7 days of treatment, respectively) cells were harvested, total RNA was extracted and brain extract-mediated influences on embryonic stem cells were investigated. Initial emphasis was put on the expression analysis of developmental markers of the neuroectodermal lineage. Additionally, overall pluripotency status of stem cells was assessed by *Oct-4* expression analysis. Expression of pluripotency marker *Oct-4* and neuronal lineage markers (neural progenitor cell marker *nestin* and mature neuronal marker *MAP2)* was quantified by real time PCR, whereas marker gene expression representative of the three primary germ layers (neuroectodermal marker *Fgf5*, mesodermal marker *T brachyury* and endodermal marker *Gata6*) was examined by semi-quantitative RT-PCR. In similar time intervals, mitotic activity of stem cells was assessed by BrdU incorporation and subsequent antibody-based detection by flow cytometry. Undifferentiated CGR8 stem cells grown in fully supplemented proliferation medium and CGR8 cells cultivated in reduced serum-free medium, but without addition of brain tissue extract were used as controls and were analysed for marker expression and proliferation in the same way as experimental cultures. All gene expression data acquired is presented hereafter as multiples of

respective gene expression measured in undifferentiated CGR8 cells grown in fully supplemented medium.

3.2.1 Brain Extract Modulates the Induction of Differentiation in CGR8 Stem Cells

Withdrawal of FCS from CGR8 stem cell cultures devoid of brain tissue extract led to a rapid and significant downregulation of pluripotency marker *Oct-4* in a time-dependent manner (day 3: 1.0 ± 0.3; day 7: 0.34 ± 0.03). Thus, serum-free culture seemed to induce differentiation processes in embryonic stem cells. In contrast, mRNA levels of *Oct-4* in CGR8 stem cells conditioned with either trauma extract (TE) or normal brain extract (NBE) did not exhibit any time-dependent dynamics, remaining at a rather constant level during the whole duration of the experiment. However, *Oct-4* levels on day 3 and 7 of tissue extract treatment were significantly lower than *Oct-4* expression level in undifferentiated stem cells (Figure 20A), suggesting the onset but subsequent stagnation of differentiation events. No significant differences in *Oct-4* expression were detected between TE- and NBE-treated cells. Thus, brain tissue extract of both normal or traumatised rat brain seemed to inhibit differentiation of embryonic stem cells, as evidenced by significant differences between *Oct-4* mRNA levels of stem cells from serum-free and brain extract-treated cultures on day 7 (control: 0.34 ± 0.03, TE: 0.76 ± 0.02; NBE: 0.70 ± 0.05) (Figure 20A). Subsequently, neural lineage commitment of ES cells was assessed by expression analysis of distinct developmental markers.

Primitive ectodermal marker *Fgf5* was not detected in undifferentiated CGR8 embryonic stem cells; therefore, experimental data represents actual amounts of PCR products measured. Expression of *Fgf5* was significantly increased in all experimental groups on day 3 (Figure 20B). Thereafter, a significant time-dependent decrease could be observed in serum-free as well as in tissue extract-treated stem cell cultures over a 7 day period (Figure 20B). No significant differences in *Fgf5* levels were observed between individual experimental groups. Initial upregulation of ectodermal marker *Fgf5*, followed by pronounced downregulation over observation period, suggested progressive neuronal lineage commitment of stem cells. However, upregulation and subsequent downregulation of *Fgf5* was strongest in serum-free stem cell culture and in stem cells treated with NBE (Figure 20B), indicating that trauma-related parameters delayed lineage commitment processes in CGR8 stem cells.

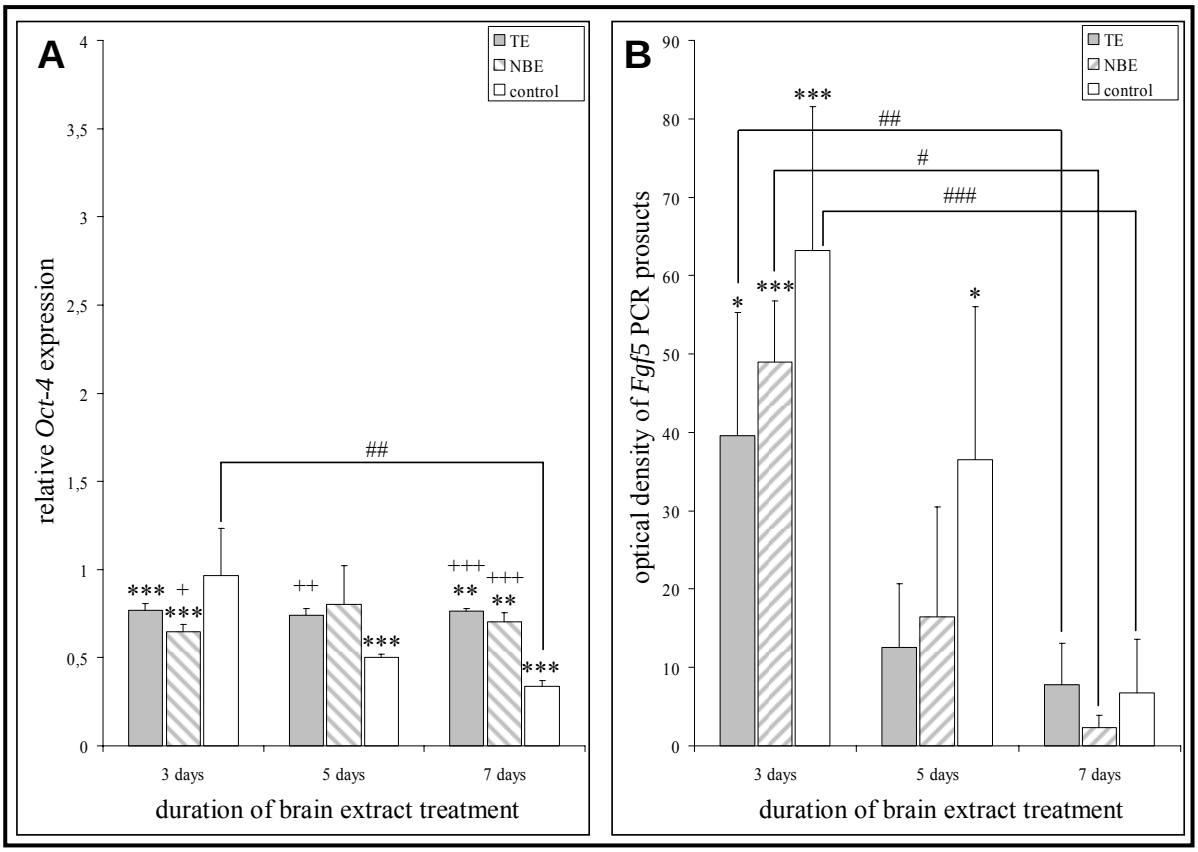


Figure 20 Brain extract-induced modulation of ES cell differentiation

mRNA expression of *Oct-4* (A) and Fgf5 (B) was assessed in CGR8 embryonic stem cells treated with trauma extract (TE) or with normal brain extract (NBE cells). CGR8 stem cells grown in serum-free medium and undifferentiated CGR8 stem cells were used as controls. Downregulation of pluripotency marker *Oct-4* and neuroectodermal marker *Fgf5* was observed in CGR8 stem cells cultured under serum-free conditions. In contrast, brain extract-treated CGR8 stem cells exhibited sustained *Oct-4* mRNA levels, although declining *Fgf5* expression was detected as well. Data is presented as mean ± SEM and represents relative amounts of gene expression compared to undifferentiated CGR8 stem cells (A) and absolute optical density of PCR products (B) (no detectable expression of Fgf5 was found in undifferentiated CGR8 stem cells), respectively. Significance vs. undifferentiated CGR8 stem cells * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significance vs. serum-free control $^{+}$ = $p < 0.05$, $^{++}$ = $p < 0.01$, $^{+++}$ = $p < 0.001$. Time-dependent dynamics $^{\#}$ = $p < 0.05$, $^{\#\#}$ = $p < 0.01$, $^{\#\#\#}$ = $p < 0.001$.

Next, mRNA levels of neural progenitor cell marker *nestin* were investigated which were significantly increased in all experimental cultures on day 3. Concurrent with downregulation of neuroectodermal marker *Fgf5* (Figure 20B), time-dependent upregulation of neural progenitor cell marker *nestin* that peaked on day 7 (3.35 ± 0.09) was detected in serum-free stem cell cultures (Figure 21A), suggesting the progressive commitment of CGR8 stem cells to a neural fate. In contrast, *nestin* mRNA levels of TE-treated stem cell culture, which were significantly increased above levels of serum-free cultured stem cells on day 3 (2.26 ± 0.01), continuously decreased during the observation time, indicating either decelerated differentiation progress or downregulation of *nestin* in favour of a more advanced neuronal cell status. NBE-treated stem cells showed no time-dependent dynamics for *nestin* expression, remaining at a significantly

elevated level compared to undifferentiated CGR8 stem cells during the complete 7-day period. This suggested the stagnation of differentiation events in NBE-conditioned stem cells. Inhibition of differentiation by brain tissue extract was clearly perceptible on day 7 when *nestin* levels of CGR8 stem cells cultured in serum-free medium (3.4 ± 0.2) had more than doubled compared to levels measured in brain extract-treated stem cells (TE: 1.05 ± 0.03, NBE: 1.5 ± 0.1) (Figure 21A).

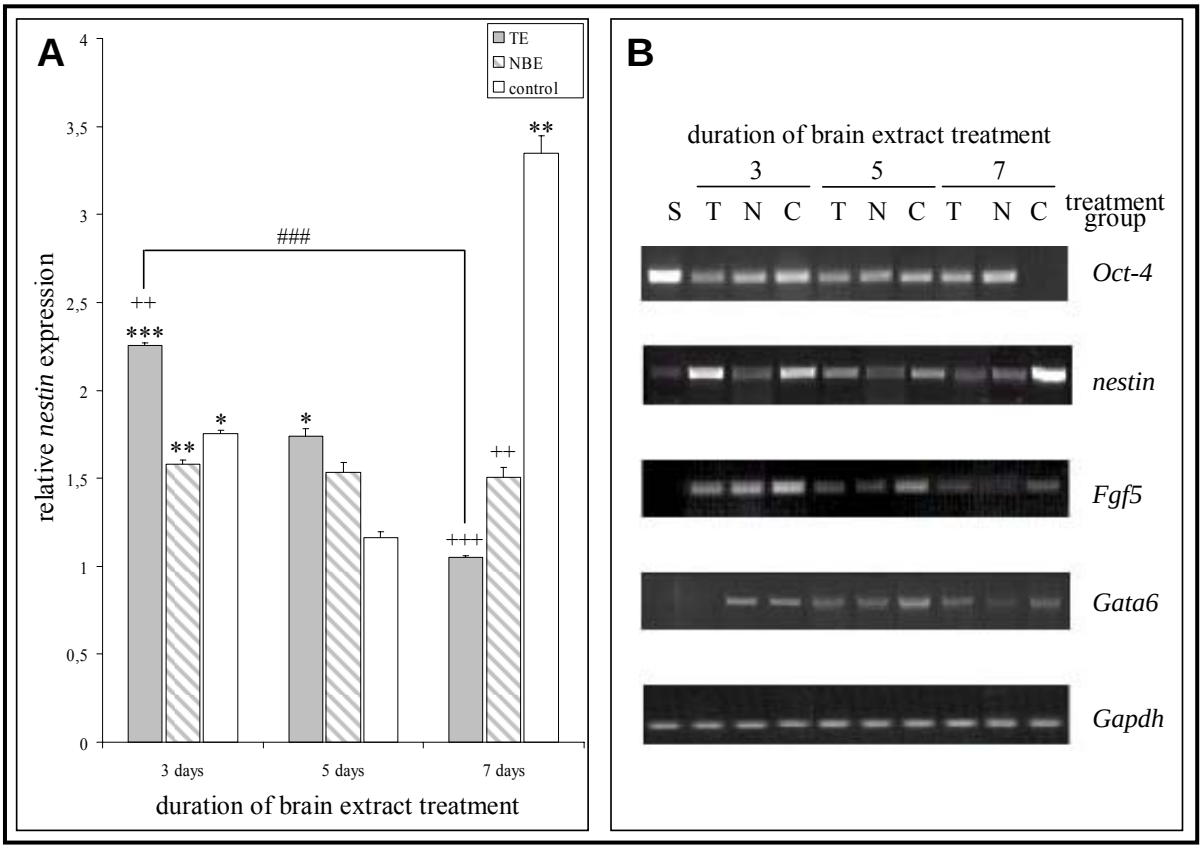


Figure 21 Expression of neural progenitor marker *nestin* by brain extract-treated ES cells

mRNA expression of *nestin* (A) was assessed in CGR8 embryonic stem cells treated with trauma extract (TE) or with normal brain extract (NBE). CGR8 stem cells grown in serum-free medium and undifferentiated CGR8 stem cells were used as controls. Upregulation of neural progenitor cell marker *nestin* was observed in CGR8 stem cells cultured under serum-free conditions, indicating neural lineage commitment of stem cells. In contrast, in TE-treated cells downregulation and in NBE-treated cells maintenance of *nestin* mRNA levels was observed, suggesting inhibition of neuronal lineage determination in ES cells by brain extract. In (B) representative gel electrophoresis of PCR products of all differentiation markers used are depicted. Data is presented as mean ± SEM and represents multiples of *nestin* expression found in undifferentiated CGR8 stem cell culture. Significance vs. undifferentiated ES cells * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significance vs. serum-free control $^{++}$ = $p < 0.01$, $^{+++}$ = $p < 0.001$. Time-dependent dynamics $^{###}$ = $p < 0.001$. (S: undifferentiated stem cells; T, TE: trauma extract treatment; N, NBE: normal brain extract treatment; C, control: serum-free control).

In order to complete analysis of the neuroectodermal developmental lineage, CGR8 stem cells of all experimental groups were also examined for the expression of mature neuronal marker *MAP2*. However, *MAP2* mRNA levels detected were so low that abundant presence of mature neuronal phenotypes in experimental cultures could be excluded (data not shown). In addition to neuronal lineage commitment, stem cell cultures were also analysed for differentiation to mesodermal and endodermal lineages. Expression of mesodermal marker *T brachyury* was not detected in any experimental group, indicating that differentiation of stem cells progressed towards other than mesodermal lineages (data not shown). Moreover, CGR8 stem cells cultures were examined for expression of *Gata6*, an endodermal lineage marker. Although *Gata6* mRNA expression was detected in experimental samples, statistical evaluation of experimental data did not reveal any tendency concerning endodermal differentiation of ES cells (data not shown).

3.2.2 Brain-Associated Factors Restrict Cell Proliferation of CGR8 Stem Cells

To determine whether brain extract has an effect on the mitotic activity of embryonic stem cells, BrdU incorporation assays were performed on brain tissue extract-treated CGR8 stem cells and CGR8 cells grown under serum-free conditions, as well as on undifferentiated CGR8 cells cultivated in fully supplemented proliferation medium. Figure 22 shows the BrdU profile of CGR8 stem cells of all treatment groups and of controls. Undifferentiated CGR8 stem cells demonstrated strongest proliferative activity of all experimental groups over 7-day period, with a peak on day 3 (54% ± 12). Subsequently, amounts of BrdU-positive undifferentiated stem cells slowly but continuously declined, possibly due to growing cell numbers and increasing cell-cell contacts, restricting further mitotic divisions. Initially displaying highest percentage of mitotically active cells of all experimental groups (59% ± 9), CGR8 stem cells grown in serum-free culture rapidly restricted mitotic divisions, evidenced by a significant decrease in the percentage of proliferating cells. On day 7 only 2% ± 2 of cells cultivated under serum-free conditions underwent mitosis (Figure 22). The significant limitation of the number of actively cycling stem cells in serum-free culture, suggested that majority of CGR8 stem cells exited cell cycle, possibly in order to start differentiating. In turn, the addition of FCS to stem cell culture could be regarded as proliferation-promoting factor, assisting in the maintenance of stem cell pluripotency. Brain tissue extract-treated stem cell cultures exhibited significantly lowest mitotic activity of all experimental groups (TE: 10% ± 2, NBE: 23% ± 11). While percentage of proliferating TE-treated stem cells remained more or less at constant levels, except for a peak on day 5 (Figure 22), proliferation of NBE treated cells declined within 7 days. No significant differences in proliferation were detected in stem cells conditioned with either TE or NBE.

Significant low levels of proliferating stem cells after brain extract-treatment indicated that brain extract restricted mitotic divisions of CGR8 stem cells.

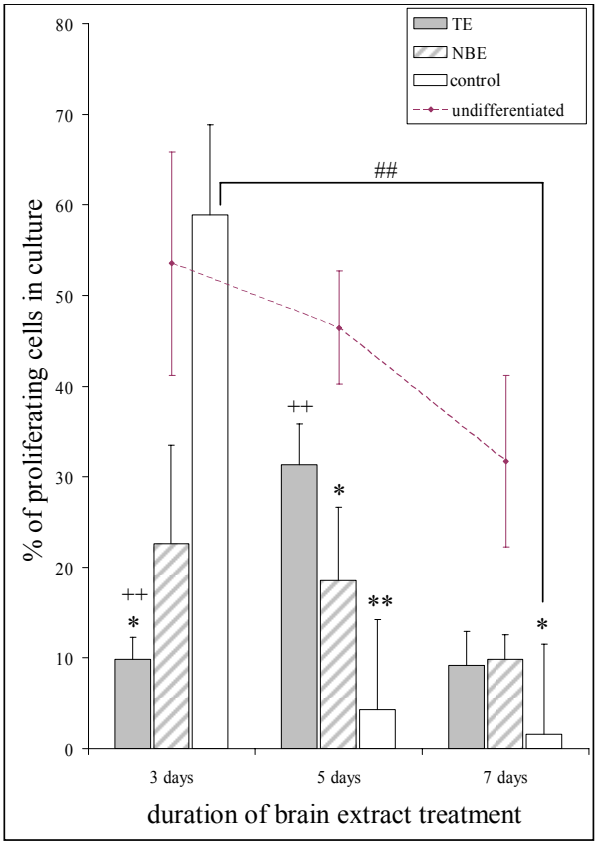


Figure 22 Percentage of proliferating brain extract-treated ES cells

Percentage of 5´-BrdU-labelled embryonic stem cells cultivated under different culture conditions was evaluated over a 7-day period. CGR8 stem cells were either treated with tissue extract derived from uninjured (NBE) or injured (TE) rat brain. In addition, CGR8 stem cells were grown in serum-free or fully supplemented proliferation medium. Undifferentiated CGR8 stem cells showed strongest proliferative activity. Number of mitotically active cells decreased significantly in stem cells cultured under serum-free conditions, indicating differentiation. Proliferation was restricted in stem cells when treated with TE or NBE. Data is presented as mean ± SEM. Significance vs. undifferentiated cells * = $p < 0.05$, ** = $p < 0.01$. Significance vs. serum-free control $^{++}$ = $p < 0.01$. Time-dependent dynamics $^{\#\#}$ = $p < 0.01$.

3.2.3 Brain Extract Initiates Distinct Changes in ES Cell's Cellular Morphology

Microscopic evaluation of stem cell morphology upon conditioning with brain tissue extract supported the results obtained by PCR and flow cytometry. Undifferentiated CGR8 stem cells grown in fully supplemented medium showed continuous proliferation activity over complete 7-day period. CGR8 stem cells initially recognisable as typical spheroid structures of compact round shape continuously expanded, finally merging with neighbouring stem cells (Figure 23). Withdrawal of FCS from culture medium not only caused reduced proliferative activity in CGR8 stem cells, but also induced morphological changes of cell phenotype. Around the edges of compact stem cell bodies cells flattened and exhibited quite distinct morphological structures (Figure 23). Following brain tissue extract-treatment, CGR8 stem cells generally showed similar behaviour with cells flattening at outskirts of spheroids. However, after NBE treatment individual stem cells separated from spheroid structures and single cells displayed small cellular outgrowths (Figure 23). In TE culture CGR8 cells grew even more insular and additionally

showed extensive axon-like sprouting (Figure 23). Moreover, it was noticed that large numbers of dead cells that had detached from well surface were found in culture supernatant of both, brain extract-treated and serum-free cultures. Because of pronounced morphological appearance of cultured cells, immunostaining for neuronal markers was conducted at day 5 of brain extract conditioning. Neuronal-like structures found in serum-free culture and in TE-treated culture stained positive for mature neuronal proteins, neuron-specific nuclear protein (NeuN) and neuron-specific enolase (NSE), indicating the differentiation of embryonic stem cells to mature neuronal phenotypes (Figure 24). Nevertheless, only few insular growing positively stained cells were detected. In undifferentiated cell culture and after NBE-treatment cells positive for NeuN or NSE were not detected (data not shown).

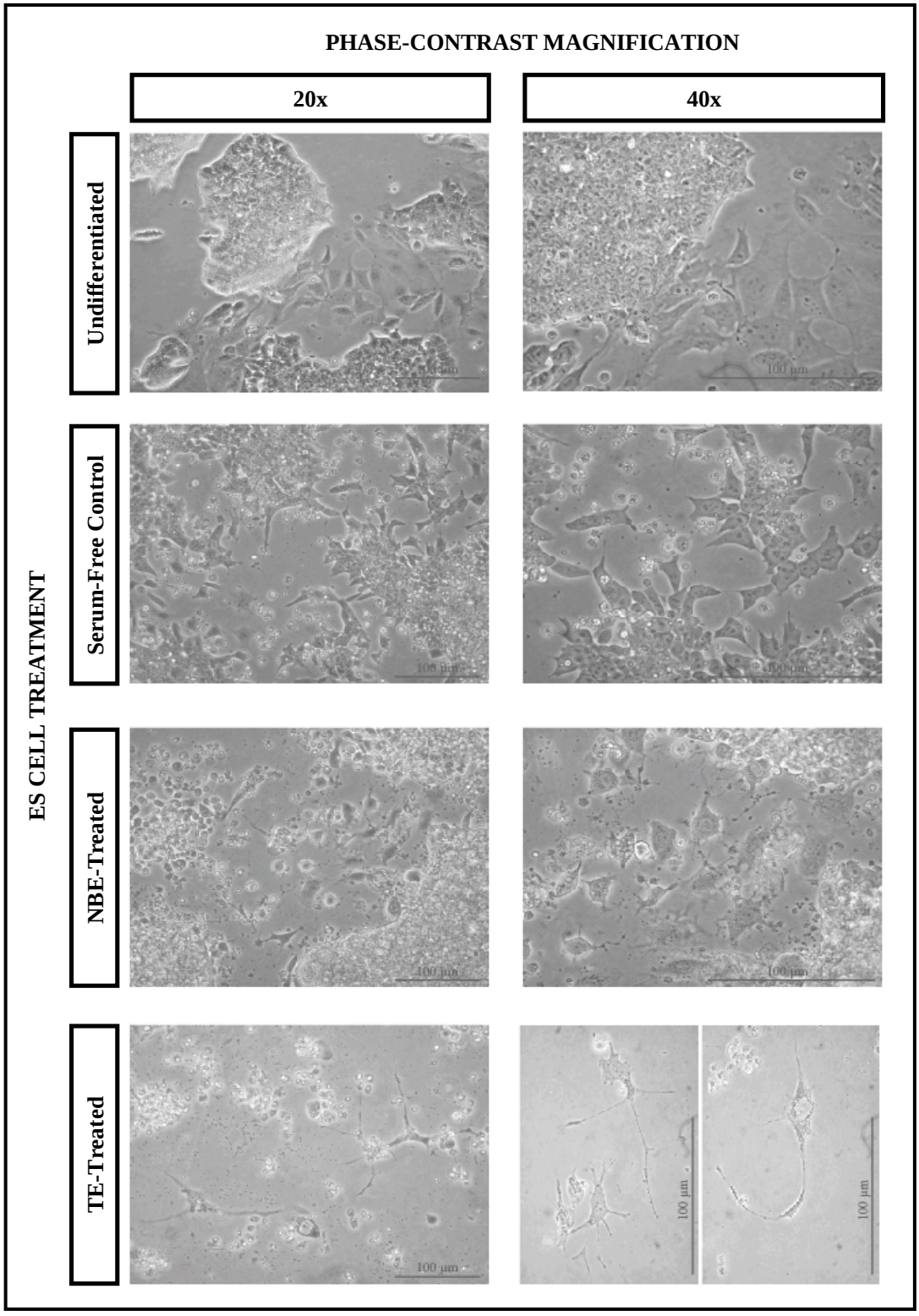


Figure 23 Changes in cell morphology following brain tissue extract treatment

In undifferentiated stem cell culture, cells continuously expanded, finally merging with neighbouring stem cells. In contrast, serum-free culture resulted in decelerated proliferation and increased differentiation processes at outskirts of aggregates. NBE- and TE-treated cells displayed enhanced differentiation activity. Cells separated from cellular spheroids and exhibited quite distinct neuronal-like morphologies. Photographs were taken after 3-day brain extract treatment with a Nikon Coolpix 5000 mounted on a Nikon Eclipse TS100 inverted microscope. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

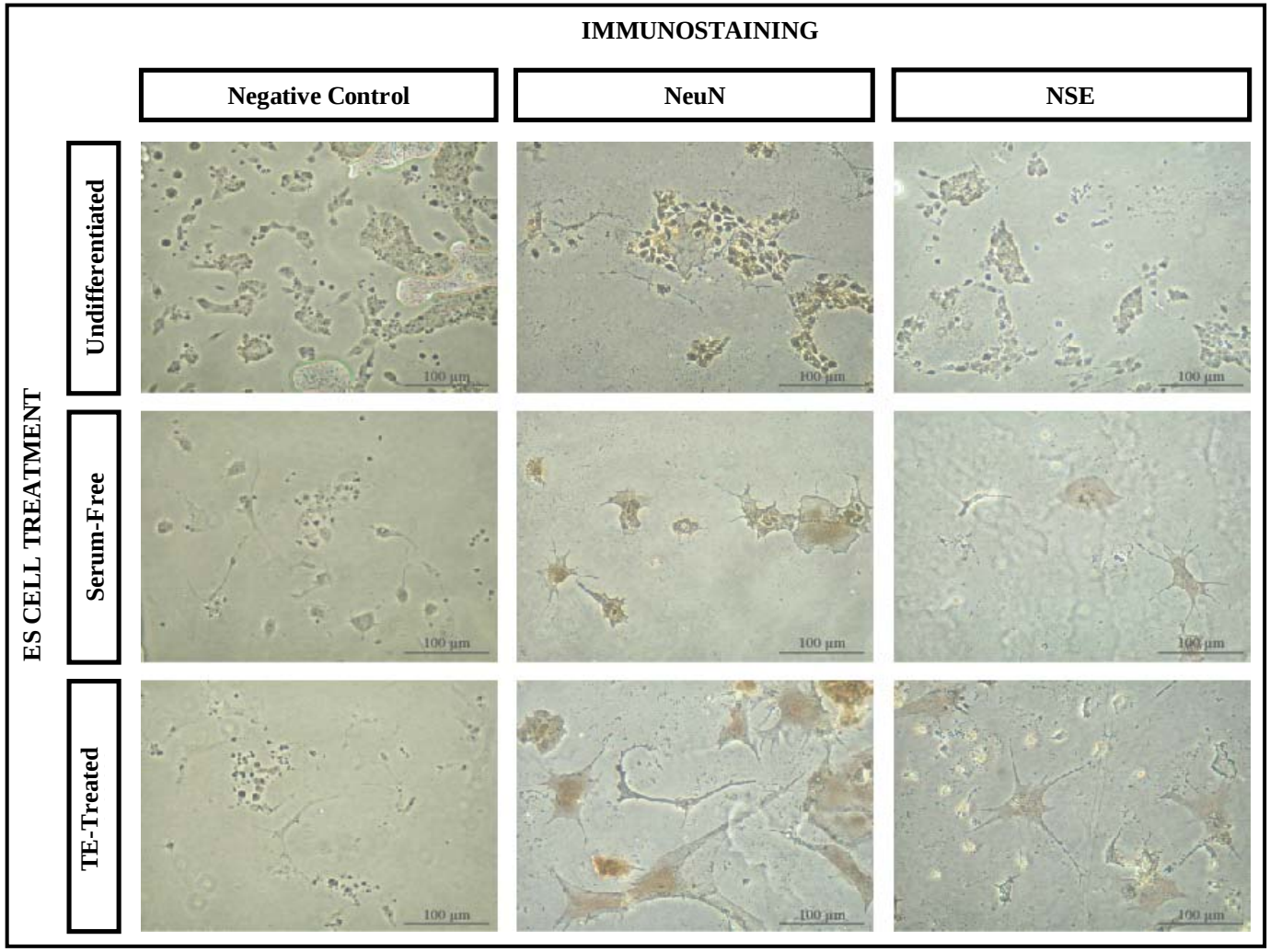


Figure 24 Immunostaining of neuronal-like structures after brain extract-treatment

Following TE treatment, individual stem cells rapidly developed neuronal-like structures with elongated processes. Cells grew insular and large numbers of cells died and detached from well surface. Distinct morphological structures found in serum-free and TE-treated ES cell culture stained positive for NeuN and NSE. Both are proteins expressed by mature neuronal phenotypes. In undifferentiated ES cell culture and in ES cells following NBE-treatment respective neuronal structures were not detected. Photographs were taken at day 5 of brain extract treatment with a Nikon Coolpix 5000 mounted on a Nikon Eclipse TS100 inverted microscope. Colour scheme of photographs was adjusted using Adobe Photoshop 7.0.

3.3 Cerebral Microenvironment Induces BDNF, but Inhibits NGF and NT-3 Release

As demonstrated before the aetiology of an acute traumatic brain injury has crucial implications for stem cells transplanted into this inhospitable environment. The small number of surviving cells, even if they differentiate to functional neurons, cannot replenish the vast amount of cells destroyed following injury. Nor can stem cell-based cell replacement be made responsible for the motor functional recovery observed in previously mentioned *in vivo* study (Riess, unpublished data). Other mechanisms, such as the possible induction of endogenous regenerative processes by transplanted cells underlying the observed outcome improvements have to be investigated. Only recently, grafted bone marrow stromal cells were shown to mediate beneficial effects via the release of neurotrophic factors (Chen *et al.*, 2005; Chen *et al.*, 2002a). Therefore, the same *in vitro* model system as in the preceding experiment was used to examine the possibility of neurotrophin release by embryonic stem cells. This time two different embryonic stem cell lines were compared; the feeder-free embryonic stem cell line CGR8 and feeder-dependent, GFP-transfected embryonic stem cell line BAC7, which was originally used for *in vivo* transplantation studies (Molcanyi, Riess, unpublished data). Again, both cell lines were cultivated in 20% tissue extracts of either uninjured or injured rat brain for up to 10 days. After 3, 5, 7 and 10 days, respectively, cell culture supernatant was aspirated and subjected to antigen detection procedures via ELISA. Stem cell lines were examined for the time-dependent release of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3). Prior to the addition to cultured cells, concentration of BDNF, NGF and NT-3, respectively was determined in brain tissue extracts alone and subtracted from experimental measurements thereafter. Stem cell lines cultivated in medium without brain extract supplementation were used as controls. In order to evaluate the effect of feeder cells on release of neurotrophic factors by stem cells, brain extract was also added to cultured feeder cells in the absence of embryonic stem cells.

3.3.1 Tissue Extract Induces Stem Cells and Fibroblasts to Produce BDNF

A time-dependent BDNF release that peaked at day 5 (67 pg/mL ± 12) was observed when BAC7 cells were cultured in serum-free DMEM/Ham's F12 devoid of brain extract (Figure 25). In BAC7 cells conditioned with brain extract derived from either traumatised (TE) or healthy rat brains (NBE) a significant increase of BDNF release into cell culture medium was detected within 3 days. Subsequently, a slow time-dependent decrease of BDNF levels was observed (Figure 25). However, BDNF release from conditioned BAC7 cells remained significantly elevated above BDNF levels of untreated cell culture for 7 days. As stem cell line BAC7 was

cultivated on a feeder cell layer, BDNF release by feeder cells was analysed independently, in order to analyse the contribution of feeder cells to BDNF levels in supernatant. Culture supernatant of untreated feeder cells contained only insignificant amounts of BDNF independent of observation time. But, when treated with TE or NBE the amount of BDNF in cell culture supernatant increased considerably with a peak at day 3 (TE: 71 pg/mL ± 72, NBE 67 pg/mL ± 56) (Figure 25). Peak BDNF levels released by feeder cells into supernatant observed were comparable to BDNF levels observed in supernatant of conditioned BAC7 cells. These observations indicated that the significant and rapid increase of BDNF levels following addition of brain extract to BAC7 cells was partly due to the activation of feeder cells.

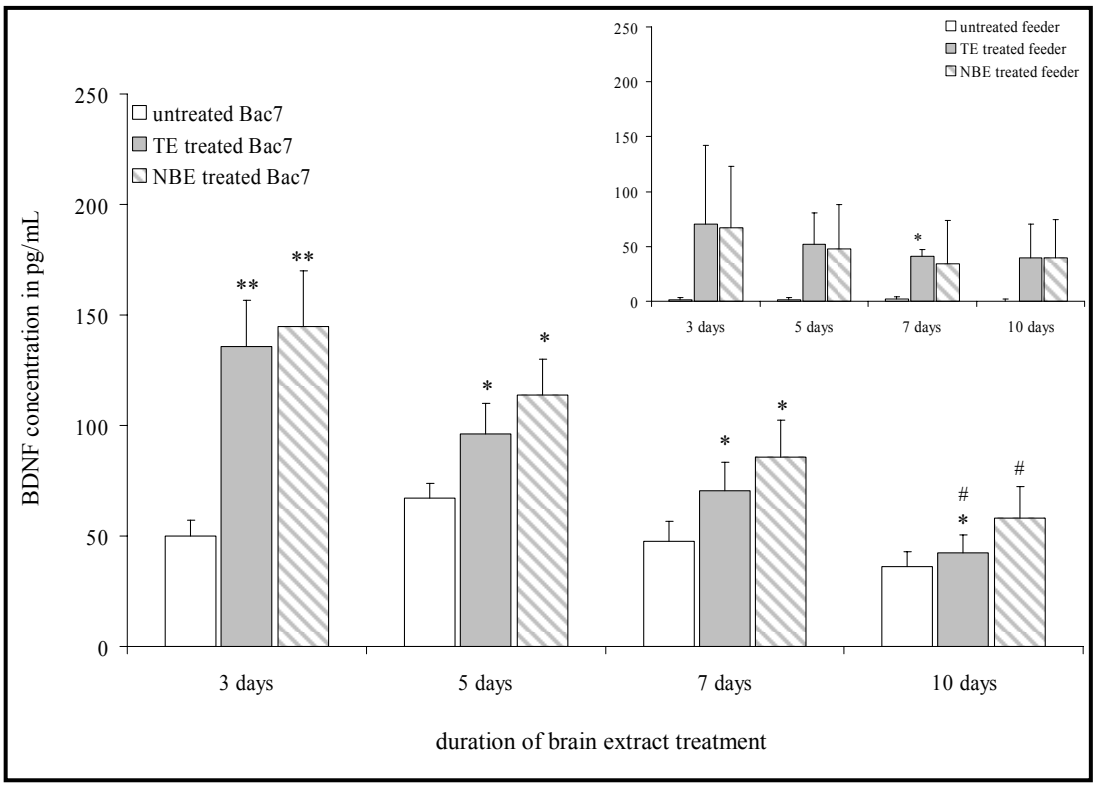


Figure 25 BDNF production by BAC7 embryonic stem cells

BAC7 embryonic stem cells showed time-dependent release of BDNF into culture medium, which was significantly augmented following brain extract treatment. Increase in BDNF release was possibly due to activation of fibroblast feeder layer. BAC7 cells were seeded onto MEF feeder layer, necessary for maintenance of stem cell self-renewal and pluripotency. Data is presented as mean ± SEM. Significance vs. untreated BAC7 stem cells/ feeder cells * = $p < 0.05$, ** = $p < 0.01$. Time-dependent dynamics $^{\#}$ = $p < 0.05$.

Comparatively low levels of BDNF were found in supernatant of CGR8 embryonic stem cells after treatment with trauma or normal brain extract (Figure 26). Following a peak at day 5, release of BDNF subsided. After 10 days no BDNF was detected in cell culture supernatant. At day 3 BDNF release was significantly augmented in cells treated with brain extract derived from

injured (39 pg/mL ± 7) and uninjured rat brains (31 pg/mL ± 5). Untreated CGR8 cells showed elevated BDNF levels after 3 days, which increased further on day 5, but remained below activated CGR8 cells.

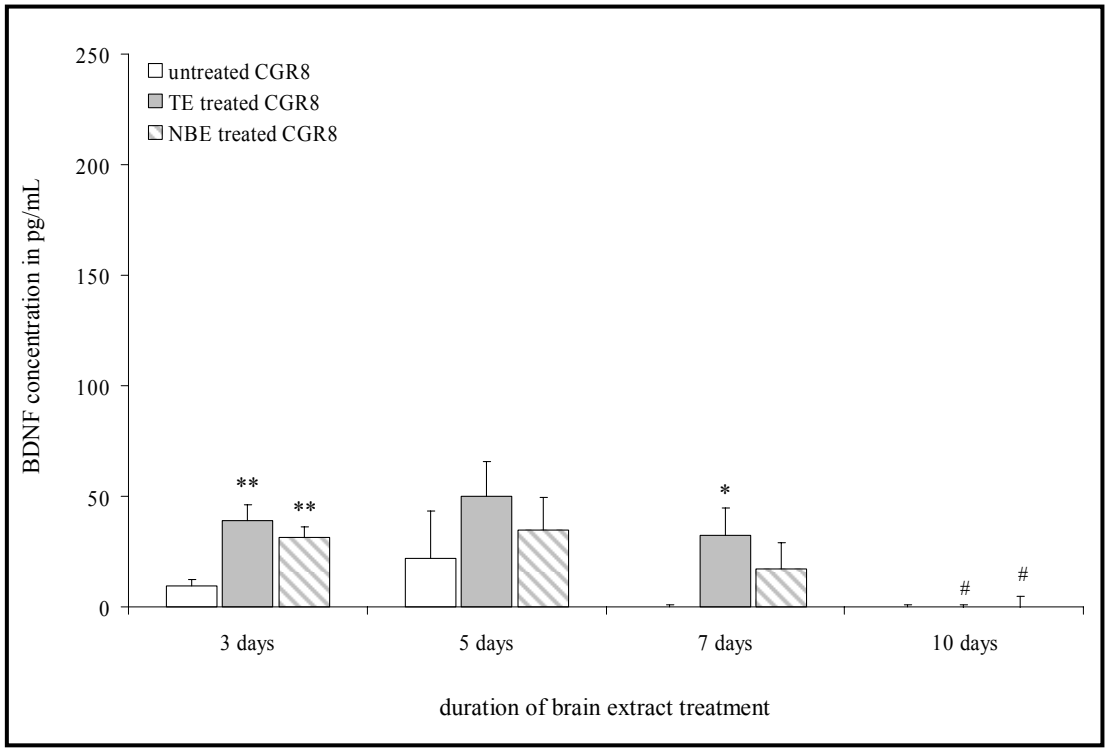


Figure 26 BDNF production by CGR8 embryonic stem cells

Untreated CGR8 stem cells showed only insignificant release of BDNF into culture medium which ceased after 5 days. Following brain extract treatment, BDNF production slightly increased, peaked at day 5, but diminished again thereafter. Data is presented as mean ± SEM. Significance vs. untreated cells * = $p < 0.05$, ** = $p < 0.01$. Time-dependent dynamics $^{\#}$ = $p < 0.05$.

3.3.2 NGF Release is Inhibited in Response to Tissue Extract Treatment

NGF release was only observed in BAC7 embryonic stem cells. In untreated BAC7 cells, NGF release peaked at day 7 (205 pg/mL ± 18) (Figure 27). Addition of either TE or NBE resulted in the significant reduction of NGF levels in supernatant by a factor of four at day 7 (Figure 27). There was no significant discrepancy in the effect of TE or NBE treatment on NGF release of BAC7 cells. NGF levels in supernatant of feeder cells was negligible (< 20 pg/mL), indicating that the time-dependent augmentation of NGF concentration in supernatant of untreated cells was solely due to the release of NGF by BAC7 stem cells.

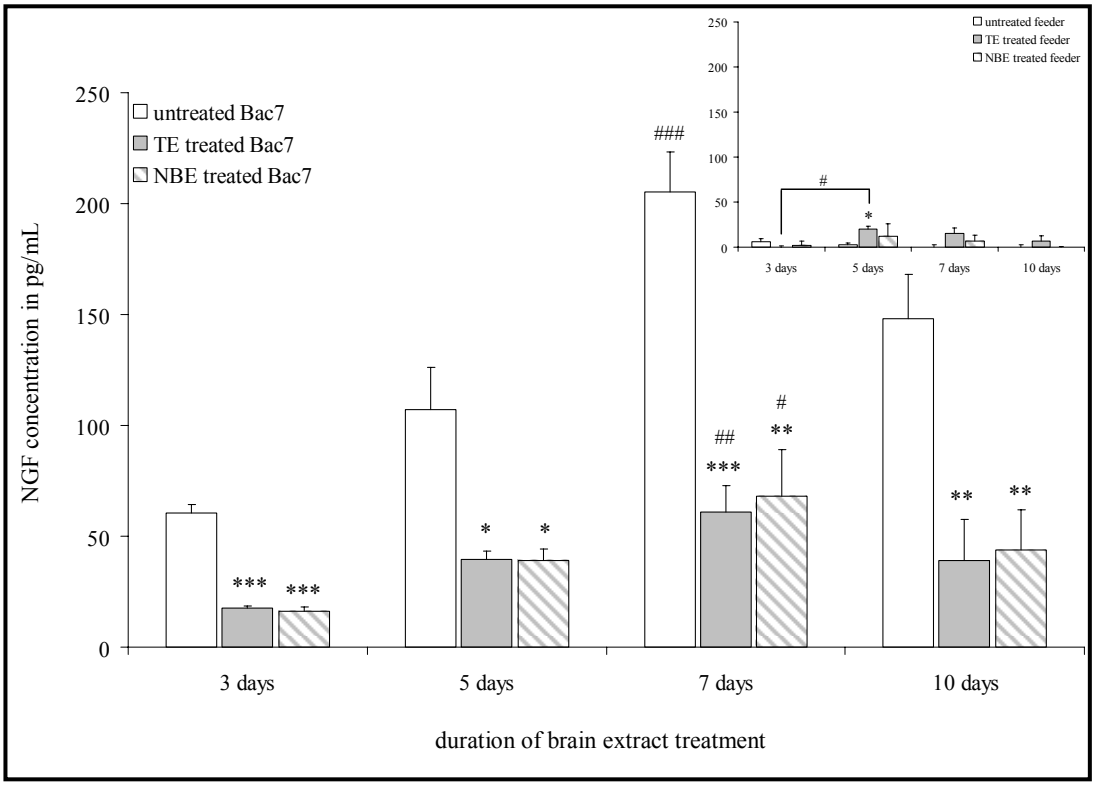


Figure 27 NGF release by BAC7 embryonic stem cells

Considerable amounts of NGF were produced by untreated BAC7 stem cells in a time-dependent manner. Treatment of BAC7 cells with trauma (TE) or normal brain extract (NBE) resulted in significant suppression of NGF release. Contribution of feeder layer to NGF production was negligible. There was no NGF release detected in CGR8 stem cell culture. Data is presented as mean ± SEM. Significance vs. untreated BAC7 stem cells/ feeder cells * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Time-dependent dynamics $^{\#} = p < 0.05$, $^{\#\#} = p < 0.01$, $^{\#\#\#} = p < 0.001$.

3.3.3 Mitotically Inactivated Fibroblasts Release NT-3

An inhibitory effect of brain extract was also observed when NT-3 levels were examined in supernatant of BAC7 cells. A peak in NT-3 concentration was observed within 3 days when BAC7 cells were cultured without brain extract (40 pg/mL ± 9). Within another 2 days, NT-3 levels in supernatant of untreated BAC7 were halved (16.3 pg/mL ± 0.8). A time-dependent decrease of NT-3 levels was observed up to day 10 (7 pg/mL ± 1) (Figure 28). Addition of TE or NBE significantly inhibited the endogenous release of NT-3 from treated cells. Brain extract-induced inhibition of NT-3 released was also time-dependent, resulting in negligible NT-3 levels at day 10 (Figure 28). There was no difference in the effect of TE- or NBE-conditioning. The time-dependent modulation of NT-3 levels observed in supernatant of untreated and TE- or NBE-conditioned BAC7 cells was almost identical to the levels observed in respective feeder cell cultures, indicating that NT-3 release had to be mainly ascribed to feeder cell activation.

In CGR8 cells, which can be cultivated in the absence of a feeder cell layer, NT-3 levels were comparatively low (< 17 pg/mL). A time-dependent decrease of NT-3 release was observed in untreated cell culture and NT-3 release ceased in both, untreated and conditioned cells at day 10. Minor differences in the effect of TE vs. NBE treatment could be observed on day 3 and 7 in CGR8 stem cell culture (Figure 28).

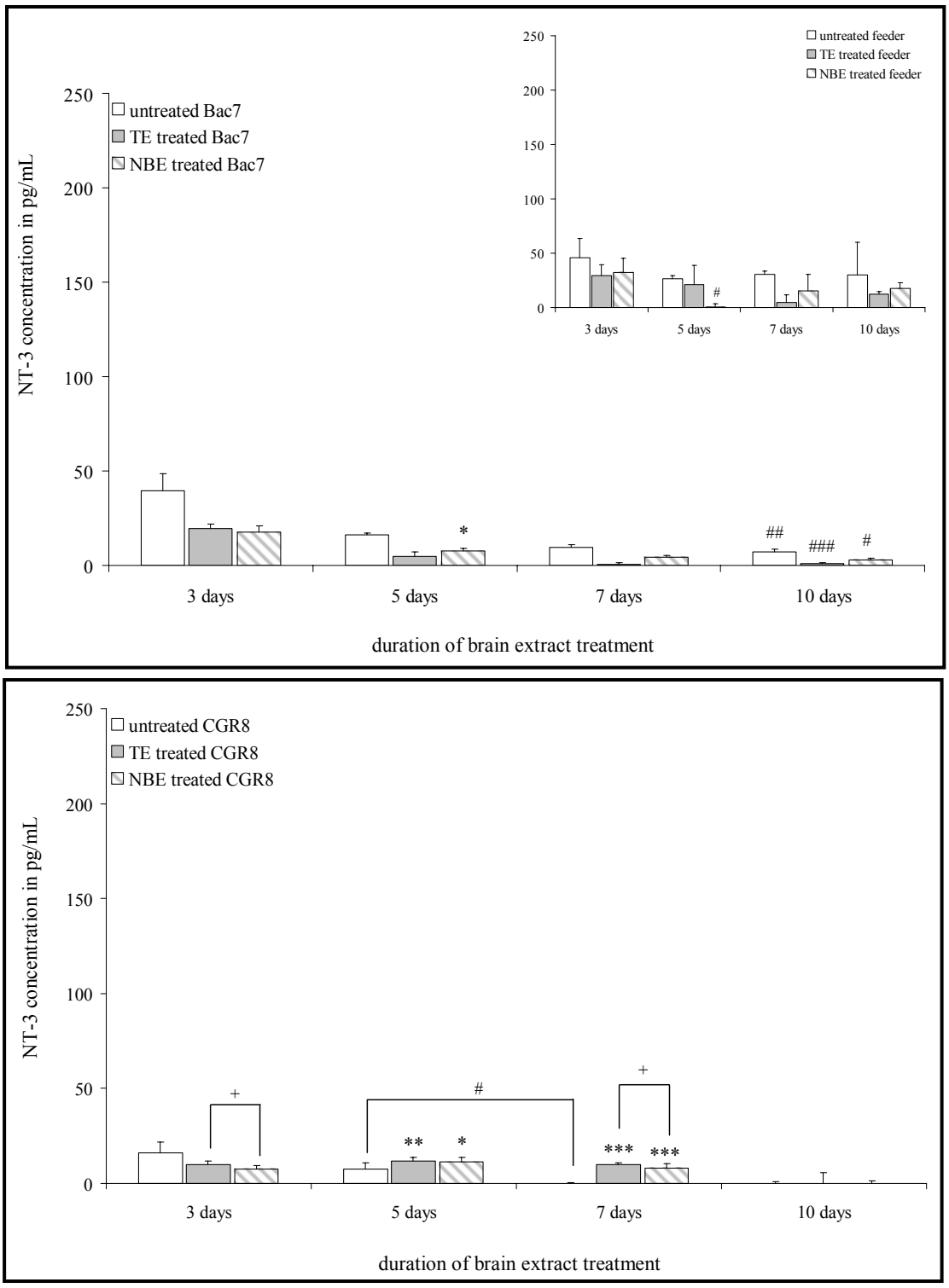


Figure 28 NT-3 release by BAC7 and CGR8 embryonic stem cells

Both, BAC7 and CGR8 stem cells released small amounts of NT-3 into culture medium. In BAC7 stem cell culture NT-3 production was suppressed following brain extract treatment. Furthermore, time-dependent NT-3 release by BAC7 stem cells corresponded to NT-3 production observed in feeder cells. This indicated that NT-3 release of BAC7 cells had to be completely attributed to feeder cell action. In contrast, NT-3 production was slightly but significantly augmented in CGR8 stem cells after brain extract treatment. Nevertheless, NT-3 concentration in culture medium ceased at day 10. Data is presented as mean ± SEM. Significance vs. untreated BAC7/ CGR8 stem cells/ feeder cells * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Significance TE vs. NBE treatment $^{+}$ = $p < 0.05$. Time-dependent dynamics $^{\#}$ = $p < 0.05$, $^{\#\#}$ = $p < 0.01$, $^{\#\#\#}$ = $p < 0.001$.

4 DISCUSSION

In view of the continuously advancing field of cell replacement strategies, embryonic stem cells increasingly gain importance. Despite successful transplantation into a variety of animal models for human degenerative diseases, the mechanisms underlying appropriate differentiation of stem cells following implantation are scarcely understood. While ES cells can readily be directed to specific cell fates and functional phenotypes *in vitro*, the respective environment encountered upon transplantation *in vivo* and its interaction with grafted cells is considered decisive for success or failure of putative cell replacement. Although paradigms for transplantation have been proposed, they are generally concerned with adult stem cell biology or were delineated from early development. Only few of them have been examined in detail *in vitro*, and even less address embryonic stem cell-based transplantation. Nonetheless, engraftment of embryonic stem cells is often the only possible approach when adult stem cells are not applicable due to the multitude of cell types lost, such as after traumatic brain injury. Generally, survival rate of grafted cells is low and reports even raised concerns about possible tumorigenity of embryonic stem cells, demonstrated by the formation of teratocarcinoma following stem cell transplantation (Bjorklund *et al.*, 2002; Erdö *et al.*, 2003). However, functional improvements following transplantation were reported, yet in the majority of studies lacking adequate evidence of graft contribution.

This doctoral thesis investigated embryonic stem cell fate in response to changing environmental conditions. Two different *in vitro* approaches were applied to monitor intrinsic ES cell properties following exogenous influences of i) endothelial cells and astrocytes, which are normally found in the neural stem cell niche *in vivo*, and ii) injury-related parameters present in cerebral tissue extracts following traumatic brain injury.

In order to evaluate status of pluripotency and possible differentiation, various genetic markers were chosen, representative for different developmental stages or lineages in ES cell life.

4.1 Developmentally Regulated Gene Expression Defines Degree of Lineage Restriction

In order to determine the developmental stage of embryonic stem cells, a variety of factors has to be considered in a multi-attribute analysis. Most stage-specific genes are transiently expressed during development, so that multiple markers have to be looked at simultaneously to roughly determine the differentiation status of embryonic stem cells. A pivotal role in maintenance of pluripotency and early lineage commitment processes of embryonic stem cells has been assigned to the transcriptional regulation of *Oct-4*, a transcription factor of the POUf1

domain. The assessment of *Oct-4* expression is essential for the determination of stem cell phenotype. Stem cell fate is governed by precise *Oct-4* transcription levels, with up- and downregulation of *Oct-4* inducing divergent developmental programmes (Niwa *et al.*, 2000; Shimozaki *et al.*, 2003). While maintenance of *Oct-4* mRNA levels within a certain range is crucial for stem cell self-renewal capacity, an initial upregulation of *Oct-4* marks the onset of differentiation events towards any of the three primary germ lineages. Conversely, immediate downregulation of *Oct-4* is associated with development of trophoectoderm. The threshold for inducing differentiation processes is apparently set at 50% above or below normal expression level found in undifferentiated cells (Niwa *et al.*, 2000). Thus, significant downregulation of *Oct-4* expression evidences advanced lineage commitment. Similarly, upregulation of *Oct-4* expression indicates onset of neuronal differentiation (Shimozaki *et al.*, 2003). Upregulation of genes specific for the three primary germ layers indicate the direction of ES cell differentiation. *Gata6,* a zinc finger-containing transcription factor, is commonly expressed during the development of extraembryonic endoderm, primitive endoderm and derivatives thereof (Fujikura *et al.*, 2002; Morrisey *et al.*, 1996). This factor is responsible for the initiation and promotion of endoderm formation. *T brachyury*, another transcription factor, is expressed in all nascent mesodermal tissue and is downregulated as these cells undergo specification towards more restricted cell lineages including skeletal muscle, cardiac muscle and connective tissue (Herrmann, 1991; Kispert and Herrmann, 1994). *Fgf*5 is transiently expressed in primitive ectoderm (Haub and Goldfarb, 1991; Hébert *et al.*, 1991), its expression representing the earliest indication of neural lineage commitment. *Fgf*5 is replaced by *nestin* during progressive neural lineage commitment. *Nestin*, an intermediate filament protein, has been demonstrated to be predominantly expressed in bipotential progenitors of the neural lineage (Dahlstrand *et al.*, 1995; Lendahl *et al.*, 1990; Sejersen and Lendahl, 1993). In particular, expression of *nestin* identifies multipotent neural stem cells of the adult CNS (Lendahl *et al.*, 1990; Sejersen and Lendahl, 1993). During the transition from a proliferating to a post-mitotic cell state, which is associated with changes in intermediate filament gene expression, a rapid decrease in *nestin* mRNA expression is observed (Dahlstrand *et al.*, 1995; Dahlstrand *et al.*, 1992). Loss of *nestin* expression is therefore associated with early events in either neuronal or glial lineage commitment (Mellodew *et al.*, 2004). Upon *nestin* downregulation stem cells still hold the multipotency to generate cellular derivatives of all three neural phenotypes, neurons, astrocytes and oligodendrocytes, repectively (Andressen *et al.*, 2001; Brannen and Sugaya, 2000). In order to determine lineage specification thereafter, expression levels of early genes within a specific cellular lineage have to be determined to give a statement of ES cell's developmental status.

Neural lineage development is thought to progress via multiple steps, involving multipotent precursor cells differentiating into more restricted precursors which then give rise to even more restricted phenotypes (Kempermann *et al.*, 2004). It is proposed that apparently homogeneous population of nestin-positive cells, able to divide and self-renew, generate more restricted precursors (Kalyani and Rao, 1998). A2B5 expression was shown to indicate oligodendroglial precursor cells of which both, oligodendrocytes and astrocytes, but not neurons, can readily be generated (Dietrich *et al.*, 2002). In contrast, neurofilament proteins (light, middle and heavy chain) are expressed in a temporal pattern during embryonic development and define different degrees of neuronal lineage commitment (Chiu *et al.*, 1995). Terminal neural differentiation has been demonstrated to involve downregulation of nestin and induction of neurofilament expression (Dahlstrand *et al.*, 1992).

Microtubule-associated protein 2 (MAP2) was chosen as a marker of terminally differentiated, fully restricted post-mitotic neurons. MAP2 is predominantly found in the lamina of neuronal somata and dendrites and its expression profile corresponds to the degree of neuronal differentiation (Sims *et al.*, 1988; Wiche *et al.*, 1983).

Genes chosen for lineage determination in this study represent only a small selection of an array of transcription factors and lineage-specific genes commonly used to verify differentiation status of ES cells. Besides, it has to be taken into account that ES cells, if not forcefully directed into one specific developmental fate, always represent rather heterogeneous cellular population with diverse phenotypes. In addition, proliferation assays and visual information acquired by microscopy are often used to emphasise data from expression analyses. Thus, interpretation of experimental data hereafter can only estimate developmental preferences.

4.2 Humoral and Cellular Environment Have Diverse Effects on Stem Cell Fate

In a first experimental approach, the functional relationship between cells, contributing to the neural stem cell niche *in vivo* and ES cells was investigated. BAC7 embryonic stem cells were co-cultured with primary and immortalised endothelial (pRBEC, SV-ARBEC) and glial cells (C6) in contact and non-contact co-culture on semi-permeable cell culture inserts, as well as cultivated in conditioned medium derived of each endothelial or glial cell type. Subsequently, expression analysis of neural lineage markers was conducted in order to determine putative milieu-dependent alterations of ES cell's gene expression profile. To assess the differentiation potential of ES cells a set of neuronal lineage genes was analysed.

4.2.1 Humoral and Cellular Cues Direct Differential Cell Fates

Expression analysis of co-cultured BAC7 embryonic stem cells demonstrated that monoculture of stem cells on collagen-coated culture inserts (used as control in co-culture experiments) resulted in predominantly neural lineage commitment of ES cells. This was evidenced by accelerated expression of early neuronal marker *neurofilament* and by increased percentage of cells expressing A2B5 antigen, which is indicative for glial progenitor cells. Simultaneously, neural precursor marker *nestin* was downregulated within 3 days. However, the influence exerted on stem cell differentiation by collagen was not as pronounced as observed after directed neuronal pre-differentiation of ES cells *in vitro.* Resulting neuronal precursor cells primarily expressed *neurofilament* mRNA evidencing the presence of premature neuronal cells. Additionally, elevated transcription of *nestin* was observed, implying that despite neuronal pre-differentiation some cells remained at multipotent progenitor cell level. More strikingly, it was demonstrated that the observed inductive effect of collagen was compensated in stem cells by the presence of endothelial cells and astrocytes in contact and non-contact co-culture. Contact and non-contact co-culture with astrocytes (C6) and immortalised brain endothelial cells (SV-ARBEC) seemed to delay cell cycle arrest and onset of differentiation events demonstrated by prolonged transcription of pluripotency marker *Oct-4.* Furthermore, neural progenitor marker *nestin* was expressed at levels close to that of undifferentiated embryonic stem cells. In comparison to stem cell monoculture on collagen, transcription of early neuronal marker *neurofilament* in ES cells seemed to be inhibited by endothelial or glial contact and non-contact co-culture. However, treatment with conditioned medium of either cell types induced significant expression of *neurofilament* and thus, suggested differentiation of stem cells towards a neuronal phenotype. These observations imply the existence of several, partially counteracting influences of conditioning cells on stem cell fate. However, somewhat surprisingly, a differential effect of contact vs. non-contact co-cultures could not be observed. The experimental setting used for contact co-cultures, Transwells™ filter inserts, has been shown to allow cell-cell contact for several cell types, including astrocyte, osteoblasts, stromal cells, and of course many neuronal cells. Astrocyte endfeet, osteoblasts and stromal villi, as well as neurites have been shown to be able to grow through the 0.4 µm pores present in these semi-permeable membranes (Hayashi *et al.*, 1997; Kawada *et al.*, 1999; Ma *et al.*, 2005; Yaccoby *et al.*, 2006) (Crouch, 2000; Smit *et al.*, 2003). Furthermore, ultrastructural studies on embryonic stem cells demonstrated that these cells too can form microvilli (Baharvand and Matthaei, 2003). However, a direct demonstration of cell-cell contacts between embryonic stem cells and co-cultured endothelial or glial cells has not been possible in this study. Thus, it cannot be excluded that direct cell-cell contacts did not exist.

This interpretation is consistent with the results obtained (no difference between contact and non-contact conditions). Further experiments will be necessary to find experimental conditions in which direct cell-cell contacts are clearly present, in order to study their influence on stem cell differentiation. However, different effects were observed between acutely conditioned medium (non-contact co-culture) and conditioned medium prepared the classic way. While classically conditioned medium seemed to induce neuronal lineage commitment of ES cells, acutely conditioned medium did not. On the other hand, both types of conditioned medium had the ability to initiate glial fates in ES cells. The observed effects should result from a different stability of the respective differentiating factors found in conditioned medium, as a contact-mediated influence can be ruled out (see above). Minimally three types of factors have to be postulated to explain these findings: an instable blocker of neuronal differentiation, a stable promoter of neuronal differentiation, and a stable promoter of glial differentiation. A further characterisation of these different factors is beyond the scope of this investigation, but may be possible by selective enrichment or purification techniques.

Discrepancies in the inductive potential of primary endothelial cells (pRBEC) and the immortalised cell line SV-ARBEC used in this study were also observed. *Oct-4* and *nestin* expression levels of stem cells co-cultured with pRBEC or grown in respective medium were elevated above those cultured with SV-ARBEC. Additionally, culture with primary endothelial cells produced higher percentages of A2B5-expressing stem cells than all other co-cultures. Incongruent results regarding different endothelial cells might be due to altered endothelial properties and the difference in cell age. Although both cell types display endothelial morphology, and express all the enzymes and transporters that are considered specific for the blood-brain barrier endothelium, immortalised cells do this at a significantly lower level (Roux and Couraud, 2005). Consequently, immortalised cell lines do not represent native endothelial cells in all respects and might therefore exert lesser or altered influences on ES cells. Moreover, the instructive potential of endothelial cells might depend on age and origin of respective cells in the same way as other cell types (Alvarez-Buylla and Lim, 2004; Sanai *et al.*, 2004; Song *et al.*, 2002). In this way, freshly isolated endothelial cells of neonatal animals might be more effective in initiating lineage commitment in ES cells than an immortalised cell line (Deli and Joo, 1996). Accordingly, co-culture of embryonic stem cells with primary endothelial cells resulted in earlier and more pronounced differentiation events. Nevertheless, it can generally be concluded that endothelial cells exert greater inductive potential on embryonic stem cells than astrocytes do.

4.2.2 Endothelial and Glial Cells Induce Glial Lineage Commitment

As demonstrated *in vitro* differentiated neuronal precursor cells clearly display properties of immature neuronal cells. Characteristics of *in vitro* differentiated precursors have earlier been described in detail (Andressen *et al.*, 2001; Arnhold *et al.*, 2000a; Arnhold *et al.*, 2000b). Embryonic stem cells grown in monoculture exhibited equally oligodendroglial progenitor and early neuronal properties, indicating the specification towards a neural cell fate induced by collagen. The inductive and versatile role of extracellular matrix (ECM) molecules, especially that of collagen on differentiation of embryonic stem cells has been reported in many *in vitro* studies (Ali *et al.*, 1998; Lin *et al.*, 2004; Ruhnke *et al.*, 2003). In these reports it has been shown that three-dimensional collagen gels supported the formation of functional synapses and neuronal circuits of neural stem and neural progenitor cells (Lin *et al.*, 2004). Furthermore, it could be shown that proliferation of cortical progenitor cells was inhibited when cells were grown on collagen IV. At the same time collagen promoted neuronal differentiation of cells. Accordingly, it was postulated that collagen as major component of the extracellular matrix seemed to be involved in the regulation of *in vivo* corticogenesis. These finding support the role of collagen in early lineage commitment of ES cells.

Regarding the inductive role of endothelial cells on stem cell fate, a study by Shen and colleagues showed that in co-culture with endothelial cells neural stem cells underwent symmetric and so, proliferative divisions to produce undifferentiated progeny maintaining stem cell multipotency. Not until endothelial cell removal later on, neural stem cells started to generate neurons, astrocyes and oligodendrocytes (Shen *et al.*, 2004). Confirming findings by Shen and colleagues, a similar effect of endothelial cells on embryonic stem cells was observed in this study. As long as endothelial cells were cultivated in close proximity of embryonic stem cells, such as in contact and non-contact co-culture, neuronal lineage commitment was inhibited. Stem cells expressed sustained levels of *Oct-4*, *nestin* and *neurofilament* close to levels found in undifferentiated stem cells. Upon removal of cells from co-culture, endothelial cell-mediated influences were restricted to humoral cues present in conditioned medium. These soluble signals seemed to induce stem cell differentiation, supported by expression of early neuronal marker *neurofilament*. Moreover, Shen and colleagues noticed predominant production of neurons within one day of endothelial cell removal, thereafter stem cells differentiated increasingly into glial lineages (Shen *et al.*, 2004). Focusing on immunocytochemical detection of markers for mature neural phenotypes, neurons (β-III-tubulin), oligodendrocytes (O4) and astrocytes (GFAP), respectively, glial progenitor cells that must have preceded mature phenotypes were not detected. Increased glial lineage commitment induced by endothelial cells was shown in the here

presented study by significantly increased numbers of A2B5-expressing cells. Taking together experimental data of both studies, neuronal differentiation of stem cells seems to precede glial lineage commitment. Rapidly evolving neuronal phenotypes are induced by humoral factors released by endothelial cells. Concomitantly, differentiation of glial precursor cells is initiated in all co-cultures. These A2B5-positive bipotential progenitors persist at a quiescent state throughout neuronal differentiation. Thus, post-mitotic neuronal phenotypes might have already evolved before cells of oligodendroglial lineages mature and develop their supportive networks.

Previous studies showed that astrocytes restrict the migration of neuroectodermal stem cells, promoting proliferation (Kornyei *et al.*, 2005). Resulting increased intercellular contacts among neuroectodermal cells in turn facilitated neuronal lineage commitment. Astroglia-conditioned medium, on the contrary, did not have an inductive effect on neuroectodermal stem cells and instead, increased cellular motility. Analogously to endothelial cells, astroglia seemed to promote proliferative rather than differentiation activities in embryonic stem cells when grown in close proximity. Supposedly, increased mitotic activity of embryonic stem cells then gradually initiates glial lineage commitment. Proliferation-induced expansion of intercellular communication has been proposed an important prerequisite for subsequent differentiation events via lateral induction/ inhibition (Chojnacki *et al.*, 2003; Grandbarbe *et al.*, 2003; Tarnok *et al.*, 2002; Tsai and McKay, 2000). It seems therefore obvious that adjacent non-stem cells initially promote the continuous proliferation of stem cells in order to establish cell-cell contacts required for subsequent differentiation (Kornyei *et al.*, 2005). Likewise, Kornyei and colleagues only used antibodies directed against mature neural phenotypes so that expression of progenitor cell markers remained undetected. Moreover, missing intermediate marker analyses has probably also led to the assumption that astroglia-conditioned medium did not exert any effects. Indeed, astroglial cells have been demonstrated to produce a variety of growth factors, which have been shown to act on stem cell proliferation and differentiation *in vitro* (Ford-Perriss *et al.*, 2001; Junier, 2000; Kilpatrick and Bartlett, 1995). The here presented data demonstrate that embryonic stem cells are indeed responsive to humoral factors produced by astroglia. Although reaction was not as pronounced as in ES cells treated with endothelial cell-conditioned medium, the amount of A2B5-positive cells was significantly increased in response to astrocyte-conditioned medium. This indicates the induction of glial lineage commitment of ES cells upon interaction with astrocyte-derived humoral factors.

4.2.3 Implantation Site Decides upon Grafted Cell Fate

The presented experimental data clearly demonstrates that self-renewal and lineage commitment of embryonic stem cells is strongly influenced by the surrounding cellular

microenvironment. Endothelial cells and astrocytes of the respective niche control stem cell fate via distinct cellular and humoral factors. The vicinity of living endothelial or glial cells leads to prolonged mitotic activity delaying differentiation events necessary for the induction of neuronal cell fates. Maintenance of ES cell's self-renewal capacity seems to be coordinated by either cell-cell mediated communication or via short-range soluble signals, both presented by adjacent endothelial or glial cells. Although sustained proliferation has previously been demonstrated to be necessary for subsequent differentiation, continuous mitotic activity has also been shown to inhibit neurogenic commitment of involved cells (Hitoshi *et al.*, 2002; Lutolf *et al.*, 2002; Solecki *et al.*, 2001). This way stem cells could possibly favour development of oligodendroglial cell fates, probably via activation of Notch signalling cascades within pluripotent stem cell population. However, interaction of embryonic stem cells with only humoral factors released by endothelial and astroglial cell types and present in conditioned medium facilitates neuronal differentiation. Hence, either the distinct gradient of a soluble signalling molecule or the absence of an easily degradable factor might act as signal for the initiation of neuronal lineage commitment. Wnt signalling during embryonic development represents one prominent example of this putative signalling mechanism. The neural crest is build by permanent recruitment of differentiating progenitor cells and follows diminishing Wnt signals; increasing distance from the source of the signal (Wnt signalling) results in mitotic arrest in progenitor cells and thus, induces differentiation (Megason and McMahon, 2002). It must therefore be concluded that stem cell fate within the cellular niche largely depends on distance-dependent variation in stem cell responsiveness to environmental cues and/ or on the differential potency of discrete gradients of soluble signalling molecules presented by adjacent endothelial and glial cells.

Transplantation strategies using embryonic stem cells thus require careful considerations regarding the choice of implantation site. Although it has been shown that stem cells are able to adapt to changing microenvironmental conditions, these findings also implicate that stem cells can alter their gene expression pattern and possibly, their cell fate in response to local inductive or restrictive cues (Hitoshi *et al.*, 2002; Shihabuddin *et al.*, 2000; Suhonen *et al.*, 1996). Hence, placed into a "wrong" microenvironment with missing or only faint cues, undesireable cell fates or even prolonged mitotic divisions, potentially causing tumor formation, could be induced in ES cells (Bjorklund *et al.*, 2002; Erdö *et al.*, 2003).

In conclusion, our findings strongly indicate that stem cell intrinsic capacities, such as proliferative activity and differentiation potential can be altered in response to exogenous cues provided by the adjacent cellular milieu. These cues can be mediated by cellular or molecular signals acting on self-renewal or lineage commitment of embryonic stem cells, resulting in

divergent cell fates. The establishment of a suitable microenvironment must thus be regarded crucial for maintenance of embryonic stem cells with neurogenetic capacity.

Appropriate environmental cues necessary for correct stem cell development are especially at risk during disease or injury. The evaluation of pathological environmental parameters on ES cell's potential to self-renew and differentiate is indispensable for successful cell replacement strategies following disease or injury.

4.3 Influence of Injury-Related Parameters on Embryonic Stem Cells

Transplantation into the specific environment of a traumatic brain injury produced various promising reports on the functional efficacy of stem cell engraftment in the past. Despite reports of functional improvements, the majority of transplanted cells were lost to the inhospitable environmental conditions following the acute phase of a traumatic brain injury. Graft function in context with observed behavioural results remained unclear. Our laboratory only recently demonstrated the phagocytosis of ES cells following transplantation into the lateral fluid percussion-injured rat brain which was surprisingly accompanied by motor functional recovery in transplant-recipients (Molcanyi, Riess, unpublished data). The second experimental approach of this thesis deals with the impact of pathological conditions triggered by traumatic brain injury on proliferation, differentiation and neurotrophin secretion of embryonic stem cells in an effort to elucidate the relationship between functional recovery and cell loss observed in *in vivo* studies.

Influence of trauma-related environmental conditions on gene expression and proliferation of ES cells was investigated by conditioning CGR8 and BAC7 embryonic stem cells with 20% cerebral tissue extract. Brain tissue extracts were derived from either uninjured or lateral fluid percussion-injured rat brains. Stem cells grown in serum-free medium and undifferentiated stem cells were used as controls. Following definite time points of tissue extract treatment (3, 5 and 7 days), CGR8 stem cells were assessed for mitotic activity, expression of neural lineage and primary germ layer markers. Additionally, over a period of 10 days, BAC7 and CGR8 embryonic stem cells, as well as MEF feeder cells were analysed for their endogenous capacity to produce neurotrophic factors and for neurotrophin release in response to cerebral tissue extract conditioning.

4.3.1 Native and Trauma-Related Parameters Induce Different Degrees of Differentiation

Examining the time-dependent influence of trauma-related, brain-derived factors on the differentiation potential of embryonic stem cells, CGR8 embryonic stem cells were conditioned with trauma extract (TE) or normal brain extract (NBE) derived from injured and uninjured rat

brain, respectively. Impact of cerebral tissue extract on proliferation and differentiation of ES cells was examined concurrently. Following serum-free culture of ES cells used as control, a time-dependent decrease in mitotic activity was demonstrated. Additionally, *Oct-4* expression was downregulated within 7 days, indicating the onset of differentiation in ES cells. In contrast, brain extract-treated stem cells displayed a restricted mitotic activity already at day 3 and prolonged *Oct-4* expression over 7 days. At this time, no significant differences between trauma extract and normal brain extract treatment were detected. Results implied that stem cell proliferation and differentiation was inhibited by brain-derived factors. Nevertheless, all stem cells, independent of treatment, exhibited initial upregulation of *Fgf5*, a representative marker of early neuroectoderm, followed by a time-dependent downregulation. Simultaneously, in ES cells of serum-free culture an upregulation of neural progenitor marker *nestin* was observed, indicating neural lineage commitment. In contrast, TE-treated cells showed a time-dependent decrease of *nestin*, implying progression of differentiation beyond progenitor cell state. Concomitant microscopic observation indicated that TE-treated CGR8 stem cells rapidly produced neuronal-like structures, characterised by elongated axon-like processes. NBE-conditioned cells displayed *nestin* at stably elevated levels over 7 days. Expression of mesodermal and endodermal developmental markers *T brachyury* and *Gata6* in treatment groups and controls was negligible, so that it can be proposed that differentiation spontaneously progressed along ectodermal lineage pathways. This is supported by the hypothesis that neuroectodermal lineage determination represents a default pathway for embryonic stem cell development (Brannen and Sugaya, 2000; Wiles and Johansson, 1997, 1999).

Immunostaining confirmed results of expression profiling. TE-treated and serum-free cultured cells stained positive for mature neuronal markers NeuN and NSE. However, cells positive for both antigens grew rather insular and isolated. Furthermore, large numbers of cells were found dead in cell culture supernatant after brain extract-treatment and serum-free culture. In undifferentiated stem cells and following NBE-conditioning, cells stained positive for NeuN and/ or NSE were not detected. Lacking evidence of mature phenotypes by *MAP2* expression analysis must be attributed to the small number of cells actually displaying post-mitotic neuronal structures.

The effect that large numbers of stem cells underwent necrosis and/ or apoptosis in both, serum-free and tissue extract-conditioned stem cell cultures is most likely due to serum depletion in culture medium. However, mitotic activity was restricted sharply in cerebral extract-conditioned cultures. This suggests either a detrimental impact of brain extract on stem cells, although cell death was also observed following healthy brain extract treatment or the forceful

induction of cell cycle exit by brain-derived factors, taking cell death into account for the sake of progressive lineage commitment. Significance of controlled apoptosis during neural development has been demonstrated where cell death is associated with fate decision, cell number control and cytoarchitecture (De la Rosa and de Pablo, 2000; Panchision and McKay, 2002). Major cell death at the expense of a small number of differentiating cells is further emphasised by the pronounced neuronal phenotypes detected in TE-treated stem cell culture. Obviously, environmental cues necessary to direct cell fate decision and maturation are present even under pathological conditions. Conversely, appropriate cues to drive differentiation seem to be missing in healthy brain extract. Lineage commitment progresses towards neural progenitor stage where advancing differentiation processes are probably paused. Initially thought to be due to a dilution of factors either by using whole hemispheres for tissue extract production or by further diluting extract to 20% working concentration, similar attenuated effects were not observed in trauma extract. Thus, it is assumed that healthy environmental conditions, not in need of cell recruitment, preserve stem cells at a ready to use, multipotential level. Differentiation to mature phenotypes shown by *in vivo* transplantation into the naïve neonatal CNS are thus most likely due to the still developing status of neonatal CNS or might be initiated in response to injury caused by the transplantation procedure.

Altogether, these data suggest that brain extract exerts ambiguous effects on embryonic stem cells, on the one hand restricting proliferative activity and differentiation potential, leading to cell death. On the other hand, cerebral trauma extract promotes the rapid and pronounced differentiation of single cells to mature neuronal phenotypes within days. However, tissue extract derived from healthy brain tissue initially induces neural lineage commitment only to suspend differentiation thereafter at progenitor cell level, possibly in order to maintain a generative stem cell pool. These *in vitro* findings are in accordance with experimental data obtained from *in vivo* studies at our laboratory. It has been shown that majority of embryonic stem cells are lost upon transplantation into the traumatically injured rat brain. Phagocytosis of implanted cells has been demonstrated by invading macrophages (Molcanyi, unpublished data). Possibly, macrophages were activated in the first place in response to apoptotic/ necrotic death of engrafted cells, and phagocytosis of stem cells is a result of primary cell death associated with trauma conditions discussed above. Despite demonstration of proper differentiation of some engrafted stem cells, survival of cells was generally rather low (Gage *et al.*, 1995; Schouten *et al.*, 2004). So far, no statistical data on implanted cells vs. surviving vs. differentiated cells is actually available. Consequently, reports demonstrating proper differentiation of engrafted cells are probably based on observations of a minority of implanted cells. This thesis for the first time presents *in vitro*

experimental data on the impact of pathologically altered environmental conditions on stem cell differentiation and proliferation. It can be concluded that few ES cells under the influence of brain-derived extract are subject to accelerated differentiation events. But, a large amount of cells can either not withstand the impact of potentially harmful environmental cues present in these extracts, or are fated to die for the sake of survival and subsequent differentiation of neighbouring stem cells. Nevertheless, the number of potentially functional phenotypes found in cell culture cannot account for the functional improvements observed in a variety of *in vivo* studies, and especially not in transplantation studies conducted by our laboratory and described previously. Alternative mechanisms, possibly activation of endogenous regenerative processes within the brain must be considered to explain stem cell-based recovery. One therapeutic concept that has proven effective in the past and might also play a putative role in functional recovery observed is the action of neurotrophic factors (Dixon *et al.*, 1997; Gharabaghi and Tatagiba, 2005; Kim *et al.*, 1996; Mocchetti and Wrathall, 1995; Sinson *et al.*, 1995).

4.3.2 Brain Extract Activates Fibroblasts to Produce Neurotrophic Factors

It is well known that neurotrophic factors, especially the nerve growth factor family, play an important role during nervous system development and a prospective role of these proteins in the recovery of function by providing trophic support after CNS trauma has been postulated (Barde, 1990; Cameron *et al.*, 1998; Klein, 1994; Mocchetti and Wrathall, 1995). Their regenerative potential in models of traumatic brain or spinal cord injuries has been delineated by a variety of investigators (Dixon *et al.*, 1987; Mocchetti and Wrathall, 1995; Philips *et al.*, 2001; Sharma, 2003; Sinson *et al.*, 1997; Sinson *et al.*, 1996; Sofroniew *et al.*, 2001; Taylor *et al.*, 2004). Only recently, Chen and colleagues showed that human bone marrow stromal cells respond to trauma extract treatment with growth factor production (Chen *et al.*, 2002a). Furthermore, they could demonstrate that marrow stromal cells following transplantation into the injured mouse brain increased NGF levels in cerebrospinal fluid attenuating neuronal loss (Chen *et al.*, 2005). These findings prompted us, in addition to gene expression analysis, to evaluate the possibility of trophic factor release by embryonic stem cells as possible explanation for functional recovery observed after ES cell transplantation into an animal model of traumatic brain injury (Riess, unpublished data). Following above experimental setting, feeder-dependent BAC7 and feeder-free CGR8 embryonic stem cells, as well as feeder cell cultures alone were conditioned with brain extracts and culture supernatants were analysed for BDNF, NGF and NT-3 release after 3, 5, 7 and 10 days of treatment. It was shown that BAC7 cells naturally secreted factors of the neurotrophin family, mainly BDNF and NGF in a time-dependent manner. Concentration of NT-3 found in BAC7 culture supernatant was rather small compared to levels measured for BDNF

and NGF. Furthermore, NT-3 was obviously produced by feeder cells in a time-dependent manner. Feeder cell, which are commonly seeded underneath stem cell cultures, are necessary for stem cell maintenance. Upon brain extract treatment, a significant augmentation of BDNF levels in culture medium of BAC7 cells was observed. However, brain extract-induced increase above endogenous production levels had to be also attributed mostly to activation of feeder cells, which initially did not produce any BDNF. In contrast, NGF levels, which originally peaked at day 7, were suppressed by brain extract conditioning. Similarly, NT-3 release of feeder cells was reduced following brain extract treatment.

CGR8 stem cells, which could be cultivated without feeder layer, showed only minor amounts of BDNF and NGF production, which slightly but significantly increased after brain tissue extract conditioning. However, in both cases endogenous growth factor production ceased after 5 days and induced growth factor release faded after 7 days in culture. NGF was not produced by CGR8 cell at all.

Interestingly, different ES cell lines demonstrated broad variation in their endogenous capacity to produce neurotrophic factors. Furthermore, ES cell lines responded differentially to microenvironmental cues provided by cerebral tissue extract by either enhancing or restricting neurotrophin production. Moreover, it could be demonstrated that in BAC7 ES cell cultures, which were originally used for transplantation experiments (Molcanyi, Riess, unpublished data), fibroblasts contributed to the majority of neurotrophic factor release found in cell culture supernatant. Mitotically inactivated fibroblast formed the basis of feeder layers necessary for maintenance of BAC7 stem cell culture *in vitro*. Endogenous as well as brain extract-induced reduction of NT-3 release in BAC7 culture could be completely attributed to feeder cells. Moreover, brain extract activated BDNF production by feeder cells, further increasing concentrations above endogenous levels. Only NGF production could exclusively be assigned to BAC7 embryonic stem cells.

Furthermore, all tested cell culture conditions displayed declining growth factor release in a time-dependent manner and generally, no differences in the influence of injured vs. uninjured brain extract were detected.

Altogether, endogenous NGF release by BAC7 stem cells and activation of fibroblasts to produce BDNF by brain extract conditioning represent possible candidate mechanisms for supporting recovery of function in *in vivo* animal models.

4.3.3 Neurotrophic Strategies Attenuate Injury-Effects *in Vivo*

The potential pharmacological use of proteins of the neurotrophin family has been discussed in detail and attempts were undertaken to rescue nervous tissue from degenerative cell death by

applying neurothrophic strategies. Sharma showed that the topical application of BDNF exhibited a protective effect after spinal cord trauma by attenuating microvascular permeability and axonal injury (Sharma, 2003). In another study, Canudas and colleagues observed an endogenous upregulation of BDNF after striatal excitotoxic injury, thus exerting a selective protective effect on dopaminergic neurons (Canudas *et al.*, 2005). And it was possible to functionally recover auditory nerve tissue after axotomy by administrating NT-3 intrathecally (Gharabaghi and Tatagiba, 2005). Moreover, it has been reported that neuronal survival was supported by intraparenchymal or intracerebroventricular administration of NGF and cognitive function was significantly improved following brain injury in rats (Dixon *et al.*, 1997; Sinson *et al.*, 1997; Sinson *et al.*, 1995, 1996). Other approaches achieved substantial success utilising genetically engineered cell lines secreting neurotrophins in order to unfold their neuroprotective abilities right at the site of injury. Transplantation of engineered (stem) cells resulted in recovery from cognitive dysfunction, promoted locomotor function and attenuated cell loss (Mitsui *et al.*, 2005; Philips *et al.*, 2001). Actually, a 1996 study demonstrated significant effects following fibroblast transplantation into the injured spinal cord (Kim *et al.*, 1996). Although originally investigating behavioural effects following transplantation of fibroblast genetically engineered to secrete NGF or BDNF, eventually, effects by modified fibroblasts matched functional improvements mediated by native fibroblasts. Generally, the transplantation of fibroblasts, even if genetically engineered, is a common method to attenuate functional deficits especially following spinal cord trauma (Jin *et al.*, 2002; Mitsui *et al.*, 2005; Tobias *et al.*, 2003). A study evaluating the potential effect of native fibroblast transplantation has not been conducted yet.

The data presented in this thesis suggest that embryonic stem cells have the intrinsic capacity to produce neurotrophic factors and cues present in brain tissue extract can be either inductive or repressive for neurotrophin production. Thus, the presence of stem cells in the brain, even if only for a short time span, might be enough to significantly enhance the concentration of neurotrophic factors in brain tissue and thus, possibly stimulate endogenous regenerative processes. What has not been evaluated in the past is the potential co-transplantation of fibroblasts as components of stem cell feeder layers. Preparation of stem cells for transplantation cogently involves detachment of feeder cells by trypsination and subsequent resuspension together with the transplant population. Thus, fibroblasts transplanted accidentally and releasing neurotrophic factors following implantation could represent a possible source for protective and regenerative effects observed.

Thus, it must be concluded that supply of trophic support by engrafted cells rather than cellular replacement via differentiating stem cells might be responsible for functional recovery

observed following stem cell transplantation into animal models of traumatic brain injury. The source of neurotrophin release following engraftment either by embryonic stem cells or by co-transplanted fibroblasts remains to be determined. Nevertheless, cellular transplantation could represent a first impulse in a cascade of events, stimulating the production of neuroprotective substances or even the differentiation of residing progenitor cells leading to a confinement of neuronal damage in the brain. Consequently, neurological dysfunction can be attenuated and/ or functional restoration of neuronal circuits is stimulated and could perhaps account for functional recovery observed following ES cell transplantation.

4.4 Considerations Regarding Choice of Model System, Cells and Culture Conditions

Relevant animal and cell culture models are an important prerequisite for standardised examinations of specific disease patterns and respective therapeutic intervention. While animal models closely simulate human disease patterns under physiological and pathophysiological conditions, cell systems are modelling discrete aspects of pathophysiological dynamics. The strength of *in vitro* modelling lies in the isolation and control of variables. Standardised analysis and control of defined variables yields important information for improved design of *in vivo* models and complements results derived thereof. Therefore, cellular environments *in vitro* must resemble the *in vivo* state as closely as possible. For co-culture experiments the use of a permeable support system has proven to be a valuable tool. Not only does it allow for cell-matrix interactions and diffusion of macromolecules, signalling molecules and hormones between compartments, but by choosing an appropriate pore size, in this case 0.4 µm, it also allows for direct cell-cell communication, simultaneously inhibiting migration and thus, mixture of cell populations. Additionally, the culture of polarised cells on permeable membrane filters permits cells to feed basolaterally and thereby carry out metabolic activities in a more natural fashion.

In initial hypothesis-driven experiments analysing endothelial cell/ astrocyte - stem cell interactions it was from the beginning opted for a more advanced state of ES cell development, in this case the formation of embryoid bodies. According to various publications the arrangement of multicellular aggregates facilitated the onset of differentiation (Gurdon, 1988a, b; Mitani and Okamoto, 1989; Tsai and McKay, 2000). Moreover, during natural *in vivo* development formation of cell-cell communication is even regarded essential (Rathjen and Rathjen, 2002). In subsequent experimental considerations examining disease-related alterations in ES cell gene expression, experimental setup was inspired by actual *in vivo* experiments where undifferentiated ES cells were transplanted into the traumatically injured rat brain (Molcanyi, Riess, unpublished data). Thus, an *in vitro* model was chosen that reproduced the situation encountered *in vivo* in approximately all details.

Concerning the choice of culture medium, it was decided to employ a minimal medium in order to minimise or even eliminate any effects mediated by unknown medium components. In particular, the addition of serum to culture medium was abandoned due to its uncharacterised composition and variation in nature (Wiles and Johansson, 1999). Furthermore, serum supplementation is thought to promote proliferative events rather than differentiation processes in respective cultures. For this reason serum depletion is part of common differentiation protocols (Andressen *et al.*, 2001; Guan *et al.*, 2001; Kawasaki *et al.*, 2000).

Seeding density of stem cells and length of culture periods were chosen partly according to previous publications of well established co-culture models and differentiation protocols (Andressen *et al.*, 2001; Arnhold *et al.*, 2000b; Chen *et al.*, 2002a; Easton and Abbott, 2002; Gaillard *et al.*, 2001; Hurst and Fritz, 1996). Partly, preliminary experiments determining optimal culture conditions of individual cell lines were conducted.

4.5 Conclusion and Outlook

The findings presented in this doctoral thesis clearly demonstrate that mechanisms controlling stem cell self-renewal as opposed to differentiation are scarcely understood and seem to be far more complex and difficult to manipulate than originally thought. The integration of cell autonomous mechanism and extrinsic signals was found to be essential for controlling cell fate and contributes to cell lineage determination during early development of ES cells. Although every lineage is controlled by a unique combination of transcription factors, the surrounding extracellular milieu influences these endogenous capacities via cell-cell contact, the secretion of diffusible humoral factors and the presentation of ECM proteins. Autocrine and paracrine pathways found in specific niches of the body may therefore direct the developmental pathway of stem cells. Moreover, changing environmental conditions, especially in response to disease or injury, impact on stem cell intrinsic properties in an unpredictable way. Altered stem cell plasticity might impede stem cell-based cell replacement in the first place, or even aggravate circumstances by pursuing malignant cell fates. Furthermore, the action of neurotrophic factors in promoting endogenous regenerative processes is not to be underestimated. As long as beneficial effects mediated via growth factor application are advantageous over outcome improvements in response to actual cell replacement, basic research strategies are needed to get stem cell-based cell replacement therapy off ground. Further investigations into cellular mechanisms, extracellular signalling and endogenous processes have to be conducted before the risks of stem cell-based therapy can be kept to a minimum and stem cell therapy can be considered as predictable, safe and effective.

Of special interest for future investigations might be the role of pathologically altered environmental conditions on stem cell biology. The identification of diffusible factors involved in manipulating stem cell fate is already under way and might help to narrow down the number of humoral candidates of environment-mediated stem cell responses. A suitable microenvironment seems to be crucial for guiding self-renewal and/ or lineage commitment of stem cell pools. Fabrication of artificial implantable carrier matrices that provide a microenvironment (graft-host-interface) appropriate for both, implanted cells and host tissue could represent an upcoming challenge for tissue engineering.

In conclusion, this doctoral thesis emphasises the astonishing potential of embryonic stem cells. However, it also stresses the enormous need for research before stem cells can be taken to the clinic.

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7 DECLARATION

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Sigrun Korsching betreut worden."

Munich, 12/12/05

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