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Analysis of murine embryonic stem cells
overexpressing the extracellular matrix molecule tenascin-R
*in vitro* and after transplantation in a mouse model of
Huntington’s disease

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1. Introduction

1.1 Huntington´s disease

Huntington´s disease (HD) is a neurodegenerative disorder that is associated with a loss of inhibitory GABAergic neurons in the central nervous system. These neurons are primarily degenerated in the striatum that constitutes an important part of the basal ganglia. In addition, GABAergic neurons degenerate in other areas of the brain including the substantia nigra (pars reticulata), the subthalamic nucleus, the thalamus, the hippocampus and also in cortical areas (Vonsattel et al., 1985). Changes in the gross anatomy of patients´ brains include a shrinkage of the striatum, enlarged lateral ventricles and cortical atrophy (fig. 1).

![Figure 1: Neuropathology of Huntington´s disease.](http://pathology.mc.duke.edu/neuropath/CNSlecture4/CNSlecture4.htm)

HD is inherited in an autosomal-dominant way and is caused by a mutation in the huntingtin gene on chromosome 4 (The Huntington´s Disease Collaborative Research Group, 1993). The mutation of this gene is characterized by an increased number of cytosine-adenine-guanine (CAG)-repeats in exon 1 that comprises 6 to 35 copies of this triplet encoding the amino acid glutamine in healthy individuals. In patients with HD 36 to 120 of these triplets can be detected. There is a correlation between the number of polyglutamine repeats and the severity as well as the age at onset of the disease (Zühlke et al., 1993; Kremer et al., 1994). HD has a prevalence of 5 to 10 per 100.000 people and manifests between the age of 30 and 55 years...
but juvenile forms as well as patients with a very late onset are occasionally described. Patients with HD typically present with involuntary hyperkinetic movements of their face, fingers, feet and thorax that generalize over time and are termed *chorea*, establishing the name *Chorea Huntington* for this disease (Folstein et al., 1986). Patients also suffer from dystonia, impaired articulation and problems with swallowing. Psychiatric symptoms such as dementia, anxiety and depression are often present frequently initiating the onset of the disease. In late stages of the disease, patients become severely rigid and akinetic and usually die about 15 to 20 years after presenting with the first symptoms.

Huntingtin plays an essential role in embryogenesis and an important role later in development. Studies on constitutive huntingtin-knockout (KO) mice revealed that a complete inactivation of this gene leads to lethality at embryonic day 8.5 (E8.5) due to extensive death of ectodermal cells (Duyao et al., 1995; Zeitlin et al., 1995). Heterozygous KO-mice show reduced neurogenesis and profound malformations of the cortex and the striatum (White et al., 1997). In chimeras consisting of wildtype and huntingtin\(^{-/-}\) cells, only few huntingtin\(^{-/-}\) neurons were found in the cerebral cortex, the striatum and other basal ganglia (Reiner et al., 2001). Moreover, the conditional inactivation of huntingtin in CamkII- (calcium/calmodulin-dependent protein kinase II) positive postmitotic neurons results in a progressive cell death and neurodegeneration in areas as similarly seen in patients suffering from HD (Dragatsis et al., 2000).

The mechanisms for striatal degeneration are currently not perfectly understood. An impaired expression and transport of brain-derived neurotrophic factor (BDNF) in the brain during development and adulthood due to the presence of a mutated form of huntingtin has been proposed as a possible cause. BDNF is a neurotrophin and is mainly expressed by cortical projection neurons in which it co-localizes with huntingtin (Fusco et al., 2003). Huntingtin contributes to the expression of BDNF in the cerebral cortex (Zuccato et al., 2001) and regulates the anterograde transport of BDNF in vesicles along microtubules in axons that project to medium-sized spiny GABAergic projection neurons expressing the BDNF-receptor trkB (Jain et al., 2001; Gauthier et al., 2004). Importantly, these neurons comprise approximately 90% of all striatal neurons (Gerfen et al., 1990; Graybiel 1990), transmit information to other subcortical nuclei (Deacon et al., 1994) and are the cells that are mainly affected in HD. BDNF-signaling regulates the expression of the striatal marker proteins DARPP-32 and ARPP-21, two dopamine and cyclic adenosine monophosphate (AMP)-regulated phosphoproteins, and thus, was proposed to be important for the maturation of GABAergic medium-sized spiny projection neurons *in vitro* and *in vivo* (Ivkovic and Ehrlich,
1999). Furthermore, BDNF controls the release of glutamate at the cortico-striatal synapse (Jovanovic et al., 2000) and thus, prevents excitotoxicity in striatal neurons, a cell death mediated by an excessive activation of glutamate receptors (Bemelmans et al., 1999). In HD the trafficking of BDNF along microtubules is disturbed possibly due to an impaired huntingtin-mediated interaction between vesicle and motor proteins (Gauthier et al., 2004) and due to an aggregation of ubiquitinated huntingtin-fragments in axonal processes and axon terminals (Li et al., 2000).

Huntingtin has been shown to prevent cells from dying by inhibiting the processing of procaspase-9 (Rigamonti et al., 2001) and is a substrate for Akt, a serine/threonine kinase that activates pathways that mediate the survival of cells (Humbert et al., 2002).

In addition to these loss of function mechanisms also gain of function mechanisms leading to disturbance of biochemical pathways due to the presence of a mutated huntingtin protein have been proposed to be involved in the pathogenesis of HD (Taylor-Robinson et al., 1996). Apart from an activation of apoptotic pathways (Gervais et al., 2002) a disturbance of the glycolysis (Burke et al., 1996), a malfunction of the respiratory chain (Gu et al., 1996), the disturbance of calcium homeostasis (Tang et al., 2005) and increased oxidative stress (Jenkins et al., 1993) have been proposed to lead to increased cell death of striatal GABAergic projection neurons and other affected neurons in HD.

Considering the systemic affection of GABAergic neurons in HD it appears to be difficult to precisely unravel causative mechanisms that lead to different clinical symptoms following cellular degeneration. Nevertheless, the striatum is primarily affected in these patients and indeed, the striatum has been shown to be an important brain structure that modulates and pioneers voluntary movements and influences emotional and cognitive behaviour (Alexander et al., 1990). It consists of the caudate nucleus and the putamen which are separated by the internal capsule containing the cortico-spinal tract. The caudate nucleus and the putamen are connected with the substantia nigra (pars compacta and pars reticulata), the internal and external segments of the globus pallidus and the subthalamic nucleus together forming the basal ganglia and transmitting inhibitory information via the thalamus to different cortical areas. Thus, a disruption of this circuitry causes a hyperkinetic state in HD patients. Interestingly, the putamen is primarily involved in motor control due to its projections to the supplementary motor area and premotor cortex (Alexander and Crutcher, 1990). In contrast, the head of the caudate nucleus is interconnected with the lateral orbitofrontal cortex which mediates limbic functions such as motivation, emotion and reward and has projections to the dorsolateral prefrontal cortex involved in cognitive functions (Alexander et al., 1990).
Furthermore, the ventral striatum has been shown to mediate limbic functions by its connections with the cingulate gyrus which together could explain for the cognitive and emotional disturbances in HD patients.

The treatment of patients with HD is based on physiotherapy, ergotherapy, logopedia and on the application of medical agents such as neuroleptics for the treatment of hyperkinetic movements or antidepressants for the relief of depressive episodes. In this context, the appearance of unwanted side effects is a frequently occurring problem of the pharmacological treatment which does not stop the progression of the disease. On the other hand there are clinical approaches to apply neuroprotective agents like ethyl-icosapent, creatine and tetrabenacine to HD patients in order to slow down the progression of the disease (Tabrizi et al., 2003). Up to date, these approaches cannot prevent the progression of the disease. Alternatively, the implantation of neural cells into degenerated areas of HD patients has been proposed to be a suitable approach to replace affected neurons and thus, to restore neuronal circuitries leading to an improvement of the neurological symptoms in patients (for review see Dunnett and Rosser 2004).

In order to find such an alternative access for the treatment of HD which aims to be curative rather than symptomatic different animal models of this disease have been established. Ideally, these animal models should mimic the neuropathology in HD patients with a selective degeneration of striatal GABAergic medium-sized spiny projection neurons that contain neuropeptides such as enkephalin, substance P or dynorphin. In contrast, the remaining cholinergic and GABAergic aspiny interneurons that constitute about 10% of all striatal neurons and express parvalbumin, calretinin or somatostatin should be spared (Marin et al., 2000). Moreover, these animals should display a behavioural phenotype in order to evaluate a visible improvement of function after medical treatment.

Animal models of HD include genetic alteration of the huntingtin gene in rodents and the induction of metabolic or excitotoxic lesions in the striatum of rodents and primates.

Several transgenic mice have been established that constitutively overexpress 115-150 CAG-repeats in an exon1-fragment of the human huntingtin gene or that constitutively or inducibly overexpress other fragments of the huntingtin gene (for review see Menalled and Chesselet, 2002). The most intensively studied transgenic mouse line is the R6/2 line that shows behavioural deficits like ataxia and tremor, cognitive impairments, neuropathological hallmarks such as cytoplasmatic inclusion bodies but interestingly, only moderate striatal neurodegeneration (Mangiarini et al., 1996; Lione et al., 1999).
The application of metabolic toxins such as 3-nitropropionic acid or malonate disrupts mitochondrial energy metabolism that results in a death of striatal neurons and also mediates functional deficits in rodents and primates (Beal et al., 1993; Palfi et al., 1996). Although the striatal cell death appears to be rather non-specific these models mimic mechanisms that may reflect similar deficits in cellular metabolism observed in patients with HD (Gu et al., 1996).

Excitotoxic lesions of the striatum of rodents and primates are established by stereotaxically injecting N-Methyl-D-Aspartat (NMDA) receptor agonists such as kainic, ibotenic or quinolinic acid into the striatum of healthy animals. Among these agents, quinolinic acid (QA) has been shown to be the most specific agent that leads to a selective degeneration of striatal medium-sized GABAergic projection neurons while other neuronal subtypes including GABAergic interneurons and dopaminergic and serotonergic afferent projections are spared (Beal et al., 1991). In fact, an intrastriatal infusion of QA results in significant decrease in substance P immunoreactivity but in significant increase in the concentration of somatostatin in the striatum pointing towards the specificity of this excitotoxic agent (Beal et al., 1991). Such lesions also lead to a reactive gliosis, striatal shrinkage and an enlargement of the lateral ventricles (Beal et al., 1989b). Moreover, these lesions cause behavioural alterations like motor hyperactivity (Sanberg et al., 1989) and motor asymmetry after unilateral application to the striatum of wildtype mice (Bernreuther et al., 2006).

1.2 Cell replacement strategies in HD

For two decades different neural cell sources have been applied in animal models of HD to find and optimize a cell replacement strategy for the loss of striatal projection neurons in HD patients. A cell replacement approach can principally be applied in patients since a precise stereotaxic implantation of cells into distinct striatal areas in humans can be performed from the surgical point of view and is associated with only minor risks of severe complications of the operation. Moreover, engrafted cells do not undergo degenerative processes as seen for endogenous cells in the brains of the recipients (Freeman et al., 2000). Therefore, implanted cells might enhance cellular stability and integrity of basal ganglia circuitries and might – besides their restorative function - mediate neuroprotection of endogenous host neurons by secreting beneficial molecules such as trophic or growth factors.

It has been shown that cells have to fulfil several criteria in order to be suitable for transplantation in animal models of HD and HD patients (Dunnett and Rosser, 2007).
One important parameter is the precise neuronal differentiation of cells. These cells must obtain a GABAergic striatal-like phenotype in order to replace neurons lost to the disease. Although the differentiation into GABAergic neurons appears to be the default pathway following neuronal induction of several cell lines (Dinsmore et al., 1996; Jain et al., 2003) precise differentiation of stem cells and neural progenitor cells into striatal GABAergic neurons expressing typical marker proteins such as DARPP-32 and STEP (striatal enriched phosphates) generally occurs infrequently and in rather small numbers (Dunnet and Rosser 2007).

A second important parameter is the survival of cells over a long period after transplantation. In this context, it has been shown that the engraftment of committed precursor cells results in a better survival compared to implanting entirely differentiated neurons (Park et al., 2007). The survival of implanted cells is closely related to their integration in the host tissue including the migration of engrafted cells into the host tissue and including the establishment of synapses with host-derived neurons. These criteria will decide about the functionality of implanted cells and thus, about the efficacy of the cell replacement strategy.

Finally, the availability of cells on demand in high quality and also in high quantity as well as their use without major ethical concerns will be very important issues which have to be addressed before routinely applying these cells for transplantation in humans.

Several cell types have been applied in animal models of HD and to some extent also in HD patients that include primary fetal striatal cell assemblies, fetal neural precursor cells, adult stem cells, non-neural cell types, immortalized cell lines and differentiated murine embryonic stem cells (for review see Dunnett and Rosser, 2007).

Primary fetal striatal cell assemblies can be isolated from the fetal ganglionic eminence, the primitive striatum in the developing forebrain. It has been demonstrated that these cells can differentiate into GABAergic inhibitory neurons after intrastratal transplantation in rodents and primates (Helm et al., 1992; Clarke et al., 1994) and project into the globus pallidum and receive afferences from the cortex, thalamus and substantia nigra after transplantation (Wictorin, 1992). Such treatment resulted in a partial remission of neurological symptoms in animals (Björklund and Lindvall, 2000). This led to the application of human primary fetal cell assemblies for cell replacement therapy in HD patients in clinical trials in several centers in Europe and the USA (Philpott et al., 1997; Kopyov et al., 1998; Rosser and Dunnett, 2004; Bachoud-Lévi et al., 2006). Whereas some patients did not improve clinically in these trials other patients had a benefit from this treatment including an improvement of chorea and cognition. However, the use of primary fetal cell assemblies for cell replacement is associated
with problems such as limited availability and thus non-reliable supply of suitable donor tissues (Björklund and Lindvall 2000) and elicits major ethical concerns since up to 16 fetuses are needed in order to supply a sufficient amount of cells for one patient (Björklund 1993; Kopyov et al., 1998). In addition, cell inhomogeneity has been discussed to be a major problem since engrafted assemblies often contain the whole ganglionic eminence instead of solely the lateral ganglionic eminence that gives rise to the medium-sized GABAergic projection neurons during development (Bjorklund, 1993).

Similar ethical and technical problems also occur when using single fetal neural progenitor cells for cell replacement that can be isolated from the ganglionic eminence and other brain regions of the embryonic brain. But the advantage of this approach is the reduction of the number of donors since these cells can be expanded \textit{in vitro} prior to transplantation (Svendsen et al., 1996; for review see Dunnett and Rosser, 2007). Mouse and human neural progenitor cells have been implanted in the quinolinic acid lesion model of HD but were found to differentiate primarily into glial cells with only few cells differentiating into striatal GABAergic neurons (Eriksson et al., 2003; McBride et al., 2004; Visnyei et al., 2006).

In addition, one transplantation study has been performed on neural progenitor cells derived from the subventricular zone (SVZ) of postnatal adult rats (Vazey et al., 2006). These adult neural progenitor cells were expanded \textit{in vitro} and engrafted in a quinolinic acid lesion model of HD in which about 5% of all implanted cells acquired a phenotype of striatal GABAergic neurons.

Furthermore, non-neural cells such as Sertoli cells (Rodriguez et al., 2003), umbilical cord cells (Ende and Chen, 2001) and bone marrow-derived cells (Lescaudron et al., 2003) have been implanted in different animal models of HD and to some extent improved functional recovery of these animals. Since only poor transdifferentiation into postmitotic neurons could be observed in these grafts, it has been discussed if the effects are mediated by neuroprotective agents secreted by these implanted non-neural cells.

Other neuroprotective approaches have been performed by engrafting immortalized cell lines into rodent and primate quinolinic acid lesion models of HD that secrete neurotrophic factors such as BDNF or CNTF (ciliary-derived neurotrophic factor) or growth factors such as NGF (Nerve Growth Factor) in order to improve the survival of host striatal neurons and thus, to slow down the progression of the disease (Martinez-Serrano and Björklund, 1996; Emerich et al., 1997a and 1997b). An improvement in behavioural tasks could be observed after intrastratial delivery of CNTF in these animals (Emerich et al., 1997a and 1997b).
Furthermore, neural cells derived from in vitro-differentiated murine embryonic stem cells have been shown to survive, to integrate and to replace degenerated host-derived GABAergic neurons in the QA-animal model of HD and to mediate a beneficial effect on the behavioural performance of these animals (Dihné et al., 2006; Bernreuther et al., 2006).

1.3 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981) but can also be isolated from 8-cell stages (Wobus et al., 1991) or morulae (Eistetter, 1989). Under optimal conditions ESCs can be expanded almost indefinitely in cell culture and thereby comprise a suitable cell population for the generation of transplantable cell assemblies. Furthermore, embryonic stem cells are pluripotent cells and thus, are able to differentiate into cells of the three germ layers mesoderm, endoderm and ectoderm. In this context, it has been demonstrated that ESCs can be cultured as so-called “embryoid bodies” (EBs) that constitute aggregates of differentiating ESCs in non-adherent cultures and consist of a core of ectoderm, mesoderm and endoderm surrounded by visceral and parietal endodermal cells (Maye et al., 2000). Using this approach ESCs spontaneously differentiated into cardiogenic (Wobus et al., 1991; Maltsev et al., 1993; Maltsev et al., 1994), myogenic (Rohwedel et al., 1994), adipocytic (Dani et al., 1997), chondrogenic (Kramer et al., 2000), osteogenic (Hegert et al., 2002), hematopoetic (Schmitt et al., 1991), insulin-producing (Schröder et al., 2006), epithelial (Bagutti et al., 1996) and also neural cell types (Fraichard et al., 1995; Strübing et al., 1995). Interestingly, the differentiation of mouse ESCs into non-somatic germ cells including oocytes and sperm cells has also been achieved (Hübner et al., 2003; Kehler et al., 2005).

During in vitro-differentiation ESC-derived cells differentially express developmentally regulated genes encoding transcription factors, enzymes, receptors or ion channels thereby mimicking expression patterns that are also seen in vivo during development (Rohwedel et al., 1994; Wobus et al., 1997; Guan et al., 1999). Thus, differentiating ESCs might be a suitable source for transplantation into patients suffering from severe disorders like cardiac, diabetic or neurodegenerative diseases.

However, many different cell types appear in culture dishes when ESCs are induced to differentiate spontaneously in vitro and as a consequence, these cultures are not pure enough to be applied for cell replacement in vivo. Therefore, many different techniques have been established to direct the differentiation of ESCs into cells of a specific lineage. Directing cues
constitute growth factors, trophic factors and mitogens. Furthermore, co-culturing of ESCs with other cell lines provides appropriate extrinsic signals to induce specific differentiation. Regarding the neuroectodermal lineage several protocols have been established to select for neural progenitors and postmitotic neuronal subtypes and to enhance their number in vitro (for review see Zhang, 2006).

Firstly, the differentiation of ESCs into neural stem cells can be achieved by culturing ESCs at a low density without the support of inactivated embryonic mouse fibroblasts that are usually needed for the propagation of undifferentiated ESCs (Tropepe et al., 2001). This study showed that the acquisition of a neuronal cell identity from ESCs is regulated through a default mechanism as has been demonstrated for developing cells in vivo. Studies in Xenopus postulated that neuroectoderm will develop from ectoderm through a default mechanism given that mesodermal tissue is absent (Hemmati-Brivanlou and Melton, 1997). In line with this, the application of noggin - an antagonist of the transforming growth factor β (TGFβ) family - blocks mesoderm-mediated signaling and thus, promotes neuronal differentiation of ESCs in vitro (Pera et al., 2004; Itsykson et al., 2005).

Secondly, neural differentiation of ESCs can be induced using a stromal cell-derived inducing activity (SDIA) protocol that describes the co-culture of ESCs with the stromal cell line PA6 derived from bone marrow (Kawasaki et al., 2000). Furthermore, several protocols use growth factors, neurotrophic factors and morphogens to drive undifferentiated ESCs into neural precursor cells and postmitotic neurons. A study performed by Bain et al. in 1995 showed that a high number of neurons can be generated when culturing EBs in the presence of retinoic acid (RA). Interestingly, a combination of RA and Sonic hedgehog (Shh) leads to the differentiation of ESCs into postmitotic cholinergic motorneurons that integrate in the adult spinal cord after transplantation and even acquire functional properties in vivo (Wichterle et al., 2002). The application of fibroblast growth factor-4 (FGF-4) promotes the differentiation of ESC-derivatives into serotoninergic postmitotic neurons (Barberi et., 2003) whereas a combination of FGF-8 and Shh has been shown to drive ESCs into dopaminergic neuronal subtypes (Lee et al., 2000). By combining a modified SDIA protocol with the application of FGF-8, Shh, ascorbic acid, cAMP, TGF-β3, glial cell line-derived neurotrophic factor (GDNF) and BDNF, undifferentiated human embryonic stem cells could be very efficiently differentiated into dopaminergic neurons (Perrier et al., 2004).

Finally, the lineage selection protocol generates a high number of neural precursor cells from EB-forming ESCs in the presence of FGF-2 applying culture conditions favouring the survival and proliferation of neural precursor cells versus mesodermal and endodermal cell
types (Okabe et al., 1996). The terminal differentiation into postmitotic neurons and glial cells is subsequently induced by withdrawal of the FGF-2 mitogen. Using a modified version of this protocol about 46% of all postmitotic neurons acquired a phenotype of GABAergic neurons (Bernreuther et al., 2006).

Embryonic stem cells can be genetically modified to carry a gene knock out mutation or to overexpress a certain gene of interest. Thus, these cells have been widely used to generate gene knock out animals. For the generation of knock out animals ESCs can be electroporated with targeting vectors carrying a disrupted gene sequence (for review see Fässler et al., 1995). This disrupted gene sequence integrates into the genome by homologous recombination (Thomas and Capecchi, 1987). Knock out animals can then be generated by reinjecting the modified ESCs into blastocysts.

Furthermore, ESCs can be genetically modified with gene constructs to overexpress genes of interests and thus, to alter their differentiation into certain cell types in vitro and after transplantation into animal models of diseases. These methods include electroporation, lipofection and lentiviral transduction of undifferentiated or even differentiating cells.

Regarding the neuroectodermal lineage it has been demonstrated that overexpressing transcription factors promotes the differentiation into distinct neuronal subtypes. Mouse ESCs overexpressing the nuclear-receptor-related-factor-1 (Nurr-1) differentiated into more neural precursor cells in vitro and generated more dopaminergic neurons after transplantation in an animal model of Parkinson’s disease with a decline of functional impairment (Kim et al., 2002). Furthermore, overexpression of the transcription factor Pitx-3 in murine ESCs led to enhanced differentiation into A9 subtype dopaminergic neurons that are selectively lost in PD patients (Chung et al., 2005). Lentiviral transduction has been applied to differentiating human ESCs to overexpress both, Pitx-3 and Nurr-1 (Martinat et al., 2006). In this study, the differentiation of these human cells into dopaminergic cells was significantly enhanced also leading to an improvement of behavioural performance of Parkinsonian animals.

ESCs have also been modified to overexpress neuronal surface molecules in order to direct their differentiation into postmitotic neurons. This has been demonstrated by overexpressing the surface molecule L1 in mouse ESCs (Bernreuther et al., 2006) that is known to promote neuronal survival and neurite outgrowth (Lemmon et al., 1989; Appel et al., 1993; Chen et al., 1999) and to influence the differentiation of neural stem cells into postmitotic neurons in vitro (Dihné et al., 2003). In this study, L1-overexpressing ESCs showed an enhanced differentiation into postmitotic GABAergic neurons when transplanted in the QA-animal
model of Huntington’s disease, an increased migration into the host striatum and had a beneficial effect on the functional recovery of these animals (Bernreuther et al., 2006).

Furthermore, the transfection of differentiating ESCs with reporter constructs driven by cell type-specific promoters constituted a suitable approach to reduce heterogeneity and to remove residual tumorigenic potential of these cells due to an elimination of undifferentiated ESCs in cultures prepared for transplantation. Using sox-1-eGFP+ mouse ESCs, neuroectodermal cells expressing sox-1 and thus the enhanced green fluorescent protein (eGFP) could be purified by fluorescence-activated cell sorting (FACS) and indeed, tumor formation was abolished when transplanted into the striatum of wildtype animals (Chung et al., 2006).

Consequently, these studies show that the use of differentiated ESCs might be a suitable therapeutic approach for cell replacement in neurodegenerative diseases.

1.4 Endogenous neural precursor cells and their reaction to brain injury and cellular degeneration

As already postulated about 40 years ago, newborn neurons are continuously generated in the adult mammalian brain throughout life (Altman and Das, 1965; Altman, 1969) raising the question if these cells can contribute to the repair of damaged or degenerated brain tissue. Constitutive neurogenesis occurs in the anterior part of the subventricular zone of the lateral ventricles where neural progenitors arise and migrate tangentially along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into olfactory granule and periglomerular interneurons (Luskin 1993; Lois and Alvarez-Buylla 1994; Betarbet et al., 1996; Winner et al., 2002). The origin of these cells in the subventricular zone has been proposed to be astrocyte-like cells (B-cells) that reside in the subependymal layer and differentiate via a stage of rapidly dividing transit-amplifying cells (C-cells) into neuroblasts (A-cells) that undergo further maturation into postmitotic neurons (Doetsch et al., 1999).

Another site of constitutive neurogenesis is the subgranular layer of the hippocampal dentate gyrus from which cells migrate into the granule cell layer to become neurons that extend their axons along the mossy fibre pathway to the CA3 region of the hippocampus (Stanfield and Triece, 1988; Hastings and Gould, 1999; Brandt et al., 2003). Besides these two neurogenic areas of the mammalian forebrain - anterior SVZ and hippocampus - neural precursor cells have been described in the spinal cord and several other brain areas including the posterior SVZ, septum, optic nerve, corpus callosum, retina, hypothalamus, amygdala, brain stem and also in the striatum (Palmer et al., 1995, 1999; Weiss et al., 1996; Shihabuddin et al., 1997;
Tropepe et al., 2000; Bernier et al., 2002; Lie et al., 2002; Zhao et al., 2003). These precursor cells could to some extent be propagated and differentiated in vitro as adherent cultures (Richards et al., 1992; Palmer et al., 1995 and 1999) or as floating aggregates, termed “neurospheres” (Reynolds and Weiss, 1992).

Brain areas can be divided into neurogenic and non-neurogenic areas. Neurogenic areas including the SVZ and the hippocampus favour region-specific neuronal differentiation of neural precursor cells when implanted into these sites (Gage et al., 1995; Suhonen et al., 1996). In contrast, the implantation of such neural precursor cells into non-neurogenic sites like the striatum promotes glial rather than neuronal differentiation pointing out that the microenvironment seems to influence the cellular fate of an implanted uncommitted cell emphasizing that a cell population should possess a committed neuronal identity prior to transplantation into non-neurogenic sites such as the striatum (for review see Sohur et al., 2006).

After brain lesions residing and migrating neural precursor cells of the mammalian brain show enhanced proliferation and start migrating towards lesioned neurogenic and non-neurogenic brain areas as has been shown for epileptic (Parent et al., 2002a), ischemic (Nakatomi et al., 2002; Parent et al., 2002b), physical (Magavi et al., 2000) and excitotoxic (Tattersfield et al., 2004) lesions of hippocampal, cortical and striatal tissue. In the QA-model of HD endogenous neuroblasts migrate directly from the SVZ into the lesioned striatum and even differentiate into postmitotic neurons to a small extent (Tattersfield et al., 2004). Interestingly, a recruitment of residing neural precursor cells has also been found in brains of Huntington patients as demonstrated by an increased cell proliferation and subsequent neurogenesis in subcortical areas (Curtis et al., 2003).

Studies have been performed to enhance the generation of endogenous neural precursor cells and to promote their migration into adjacent brain areas. The intraventricular infusion of growth factors such as epidermal growth factor (EGF), FGF-2 or TGF-α increased neural precursor proliferation in the SVZ (Craig et al., 1996; Kuhn et al., 1997). Similarly, the injection the neurotrophic factor BDNF into the lateral ventricle of adult animals led to enhanced neurogenesis in the SVZ and increased number of newborn neurons in the olfactory bulbs (Zigova et al., 1998). Additionally, the treatment with BDNF induced migration of neural precursor cells from the SVZ into the septum, thalamus, hypothalamus and also the striatum (Benraiss et al., 2001; Pencea et al., 2001). Furthermore, when directly infused in the striatal parenchyma of wildtype animals TGF-α induced cell proliferation in the SVZ and
migration of these cells from the lateral ventricle towards the injection site in the adjacent striatum (Cooper and Isacson, 2004).

These approaches showed that endogenous neural precursor cells can be activated to proliferate and migrate in the adult brain. However, injected growth and neurotrophic factors diffuse and dilute with time and thus, alternative approaches have been applied like the infusion of retroviral vectors or the implantation of transfected cells that constantly secrete beneficial molecules in the surrounding tissue.

1.5 The role of tenascin-R in the central nervous system

During embryogenesis and postnatal life the specification and integrity of cells is dependent on the communication with their surrounding environment including neighbouring cells, soluble factors and the extracellular matrix (for review see Hynes and Lander, 1992). Transmembrane glycoproteins transduce signals from other cells by binding surface molecules in a homophilic or heterophilic manner and are activated or blocked by soluble molecules such as growth factors and non-soluble components of the extracellular matrix constituting proteoglycans and glycoproteins. One family of glycoproteins in the extracellular matrix are the tenascins that consist of tenascin-C (myotendinous antigen, cytotactin), tenascin-X (gene X), tenascin-W (tenascin-N) and tenascin-R (restrictin, janusin) in mammals (for review see Chiquet-Ehrismann 2004).

Tenascin-R is almost exclusively expressed in the central nervous system after birth and shows a highly dynamic spatial, temporal and cell-type specific expression pattern during postnatal life (Pesheva et al., 1989; Fuss et al., 1993). It is expressed by myelinating oligodendrocytes in the cerebellum, the olfactory bulb, the fornix and the corpus callosum, by inhibitory interneurons in the cerebellum and the hippocampus, by postmitotic neurons in the olfactory bulb, by motor neurons in the spinal cord and by horizontal cells in the retina (Fuss et al., 1993). In white matter tenascin-R is located at nodes of Ranvier, within myelin sheaths and between myelin sheaths and their axon (French-Constant et al., 1986; Bartsch et al., 1993). In grey matter tenascin-R is detectable in perineuronal nets that surround inhibitory interneurons and motorneurons and that have been shown to have neuroprotective effects on these cells (Angelov et al., 1998; Bruckner et al., 2000). Furthermore, perineuronal nets are known to modulate synaptic excitability in vitro (Dityatev et al., 2007). Interestingly, synthesis of tenascin-R by oligodendrocytes starts early in postnatal development, peaks during time of myelination 7 to 14 days after birth and is downregulated at later
developmental stages. In contrast, neurons start to synthesize tenasin-R about 7 days after birth and express it continuously during adulthood.

Similar to the other members, tenasin-R contains an N-terminal cysteine-rich region, fourteen complete and one partial epidermal growth factor-like (EGF-like) domains, nine fibronectin type III (FN III) homologous repeats and a C-terminal fibrinogen-like domain (Weller et al., 1991; Fuss et al., 1991). Tenasin-R consists of two major isoforms of 160 kDa (tenasin-R 160) and 180 kDa (tenasin-R 180) in mammals. The 160 kDa isoform lacks a portion of its amino terminal region and the sixth FN III domain due to alternative splicing (Weller et al., 1991) although the latter observation has been challenged recently (Woodworth et al., 2004). Since in the amino terminal region disulfide bonds are formed, the two isoforms show differences also on an ultrastructural level (Pesheva et al., 1989).

The expression of the different isoforms in the postnatal brain is spatially and temporally regulated (Pesheva et al., 1989). Tenasin-R 160 is found predominantly in the molecular layer of the cerebellar cortex whereas tenasin-R 180 is expressed predominantly in the white matter and the granular layer in the cerebellum. Moreover, tenasin-R 160 is only weakly expressed right after birth but is the more predominant isoform in adulthood whereas tenasin-R 180 is the first isoform to be found after birth but its expression declines during postnatal life (Pesheva et al., 1989; Woodworth et al., 2004).

As a glycoprotein tenasin-R carries N- and O-linked oligosaccharides. Besides O-linked chondroitin sulfate glycosaminoglycans tenasin-R carries N-linked carbohydrates that contain the human natural killer antigen-1 (HNK-1) and other sulfated sugar residues (Woodworth et al., 2002 and 2004). Interestingly, differently glycosylated forms of tenasin-R exist that are spatially and temporally expressed in the developing and adult cerebellum. Moreover, patterns of N-glycosylation are dependent on the cell type that secretes tenasin-R to the extracellular space (Woodworth et al., 2002 and 2004).

Several proteins have been identified to bind to tenasin-R. These proteins include the neuronal surface molecule F3/contactin and other components of the extracellular matrix such as fibronectin, aggrecan, brevican, neurocan, versican and phosphacan (for review see Jones and Jones 2000).

Some of the functions of tenasin-R have been linked to different binding sites within the FN- and EGF-like domains (for review see Jones and Jones, 2000). Since many of these functions of tenasin-R have been shown to be both promoting or inhibiting dependent on the experimental setup, tenasin-R was also named janusin adopted from the name of the Latin god Janus, the god with the two faces symbolizing ambivalence (for review see Schachner et
Both adhesive and non-adhesive functions have been described for tenascin-R dependent on the cell type used. In vitro-experiments on neurite outgrowth of neurons showed both a promoting (Husmann et al., 1992) and an inhibiting (Norenberg et al., 1995) role for tenascin-R depending on the way how tenascin-R was presented to the cells (Schachner et al., 1994; Jones and Jones, 2000). In vivo, tenascin-R serves as a repellent guidance molecule in the optic nerve in zebrafish (Becker et al, 2003). On the other hand tenascin-R mediates the detachment of migrating cells from the RMS within the mammalian olfactory bulb in order to reach their target area in the olfactory cortex establishing tenascin-R as an attracting guidance molecule in vivo (Saghathelyan et al., 2004). In this context, it has been demonstrated that transgeneic BHK-cells secreting the extracellular matrix protein tenascin-R could attract residing and migrating neuroblasts from the SVZ and the RMS when implanted into the most anterior part of the striatum or in the cerebral cortex in close proximity to the RMS (Saghateryan et al., 2004). Importantly, these residing and migrating cells have not been attracted by implanted non-transfected cells pointing out that tenascin-R was the molecule that recruited endogenous neural precursor cells from neurogenic towards non-neurogenic sites in the brain and might therefore be a beneficial candidate molecule in the context of endogenous regeneration in the lesioned mammalian central nervous system.

Interestingly, tenascin-R deficient (TNR−/−) mice do not differ in their gross anatomy from their wildtype littermates and do not show major patterning defects in areas where tenascin-R is expressed except for the olfactory bulbs where migrating cells from the RMS do not properly enter their cortical target area but are found to be stuck in the olfactory RMS (Weber et al., 1999; Saghathelyan et al., 2004). Furthermore, the morphology of perineuronal nets and the distribution of the tenascin-R binding molecule phosphacan in the extracellular matrix are changed in TNR−/− mice. In addition, structural abnormalities of synapses in the hippocampus can be observed in TNR−/− mice (Nikonenko et al., 2003) and the conduction velocity in the optic nerve is remarkably reduced in these animals (Weber et al., 1999). Finally, tenascin-R deficient mice display an increased anxiety and show impaired motor coordination compared to their wildtype littermates (Freitag et al., 2003).
2. Aims of this study

Since the treatment of neurodegenerative diseases such as Huntington’s disease can only be regarded as symptomatic a cell transplantation approach into the basal ganglia of patients might be a suitable alternative. After transplantation these cells might replace degenerated neurons and might help to re-establish damaged neuronal circuitries. Therefore, this study set out to investigate the regenerative potential of embryonic stem cells genetically manipulated to overexpress the extracellular matrix molecule tenascin-R with regard to differentiation, survival, migration, synaptogenesis, recruitment of endogenous neural precursor cells and functional recovery in a mouse model for Huntington’s disease.
3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

All chemicals were purchased from the following companies in p.a. quality, if not indicated otherwise:

Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Molecular weight standards were obtained from Invitrogen (Karlsruhe, Germany). Restriction enzymes were obtained from New England Biolabs (Frankfurt am Main, Germany). Cell culture material was ordered from Greiner (Frickenhausen, Germany) or Nunc (Roskilde, Denmark).

3.1.2 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaesthetic solution</td>
<td>20%</td>
<td>Ketanest® (25 mg/ml) (Pfizer, Karlsruhe, Germany)</td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>Rompun® (2 % solution) (Bayer, Leverkusen, Germany)</td>
</tr>
<tr>
<td>Antigen retrieval solution</td>
<td>10 mM</td>
<td>Sodium citrate in deionized water</td>
</tr>
<tr>
<td>Bis-benzimide solution</td>
<td>5 µg/ml</td>
<td>Bis-benzimide in PBS</td>
</tr>
<tr>
<td>Blocking solution</td>
<td>5% (v/v)</td>
<td>Normal goat / normal donkey serum</td>
</tr>
<tr>
<td>(Immunohistochemistry)</td>
<td>2% (v/v)</td>
<td>Triton X-100</td>
</tr>
<tr>
<td></td>
<td>0.02% (w/v)</td>
<td>NaN₃</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>5% (w/v)</td>
<td>Instant milk powder in TBST</td>
</tr>
<tr>
<td>(Western Blot)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blotting buffer</td>
<td>25 mM</td>
<td>Tris</td>
</tr>
<tr>
<td>(Western Blot)</td>
<td>192 mM</td>
<td>Glycin</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Buffer/Stock Solution</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Boston buffer</strong></td>
<td></td>
<td>Tris pH 8.0</td>
</tr>
<tr>
<td></td>
<td>50 mM</td>
<td>KCl</td>
</tr>
<tr>
<td></td>
<td>2.5 mM</td>
<td>EDTA</td>
</tr>
<tr>
<td></td>
<td>0.45%</td>
<td>NP-40</td>
</tr>
<tr>
<td></td>
<td>0.45%</td>
<td>Tween 20</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/ml</td>
<td>Proteinase K</td>
</tr>
<tr>
<td><strong>Cacodylate buffer</strong></td>
<td>0.2 M</td>
<td>Sodium cacodylate</td>
</tr>
<tr>
<td></td>
<td>0.2 M</td>
<td>HCl solution</td>
</tr>
<tr>
<td><strong>DNA-sample buffer (5x)</strong></td>
<td>20% (w/v)</td>
<td>Glycerol in TAE buffer</td>
</tr>
<tr>
<td></td>
<td>0.025% (w/v)</td>
<td>Orange G</td>
</tr>
<tr>
<td><strong>DNase-solution</strong></td>
<td>10 µl</td>
<td>DNase-stock solution (Qiagen) in 70 µl RDD-buffer</td>
</tr>
<tr>
<td><strong>dNTP-stock solutions</strong></td>
<td>20 mM each</td>
<td>dATP, dCTP, dGTP, dTTP</td>
</tr>
<tr>
<td><strong>Ethidiumbromide-staining solution</strong></td>
<td>10 µg/ml</td>
<td>Ethidiumbromide in 1xTAE</td>
</tr>
<tr>
<td><strong>Ferricyanpotassium solution</strong></td>
<td>50% (v/v)</td>
<td>20% Potassiumhexacyanoferrat (III) solution</td>
</tr>
<tr>
<td></td>
<td>50% (v/v)</td>
<td>HCL (1%)</td>
</tr>
<tr>
<td><strong>Ligation buffer (10x)</strong></td>
<td>200 mM</td>
<td>Tris-HCl, pH 7.9</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>MgCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>Dithiothreitol (DTT)</td>
</tr>
<tr>
<td></td>
<td>6 mM</td>
<td>ATP</td>
</tr>
<tr>
<td><strong>Phosphate buffered saline (PBS)</strong></td>
<td>150 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>Na₃PO₄ pH 7.4</td>
</tr>
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</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Buffer/Component</th>
<th>Volume</th>
<th>Concentration</th>
<th>Composition/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer (pH 6.9)</td>
<td>57.7 ml</td>
<td>Na$_2$HPO$_4$ (1M)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42.3 ml</td>
<td>NaH$_2$PO$_4$ (1M)</td>
<td></td>
</tr>
<tr>
<td>Protease-inhibitors</td>
<td>COMPLETE™ pills. Resuspending 1 tablet in 2 ml solution results in a 25x stock solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinolinic acid solution</td>
<td>60 mM</td>
<td>Quinolinic acid (dissolved in 0.5 M NaOH); pH adjusted to 7.4</td>
<td></td>
</tr>
<tr>
<td>RIPA-buffer (Cell lysis)</td>
<td>50 mM</td>
<td>Tris-HCl, pH 7.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 % (w/v)</td>
<td>Triton X-100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 mM</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>EGTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>Na$_3$VO$_4$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 x</td>
<td>Protease-inhibitor</td>
<td></td>
</tr>
<tr>
<td>Sample buffer (5x) (Protein-gels)</td>
<td>0.312 M</td>
<td>Tris-HCl pH 6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 % (w/v)</td>
<td>SDS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 % (w/v)</td>
<td>β-Mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 % (v/v)</td>
<td>Glycerol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.13 % (w/v)</td>
<td>Bromphenol blue</td>
<td></td>
</tr>
<tr>
<td>SDS running buffer (10x) (Protein-gels)</td>
<td>0.25 M</td>
<td>Tris-HCl, pH 8.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.92 M</td>
<td>Glycine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 M</td>
<td>SDS</td>
<td></td>
</tr>
<tr>
<td>Stripping buffer (Western blot)</td>
<td>0.5 M</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 M</td>
<td>Acetic acid</td>
<td></td>
</tr>
<tr>
<td>TAE (50x) (DNA-gels)</td>
<td>2 M</td>
<td>Tris-Acetat, pH 8.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>EDTA</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

20

TE (10x) 0,1 M Tris-HCl, pH 7.5
10 mM EDTA

Tris Buffered Saline (TBS) 10 mM Tris-HCl, pH 8.0
150 mM NaCl

TBST 10 mM Tris-HCl, pH 8.0
150 mM NaCl
0,01 % (v/v) Tween

3.1.3 Plasmids

pBlueCAM-PGK Plasmid (Stratagene; La Jolla, CA, USA) containing the promoter sequence of the murine 3-phosphoglycerokinase I (PGK). Chloramphenicol-resistance

pcDNA3.1myc-his vector Eukaryotic expression vector (Invitrogen) containing a myc-his tag in the multiple cloning site. Amp-resistance

pGEM-T-Easy-TNR Cloning vector pGEM-T-Easy (Promega; Mannheim, Germany) containing the full length cDNA of TNR kindly provided by Sandra Nickel and Dr. Nuray Akyüz. Amp-resistance

p901-PGK-neo Vector containing the neomycin phosphoriltransferase gene under the control of the PGK promoter. Original vector pKO901 was purchased from Lexicon Genetics Incorporated (The Woodlands, Texas USA). Amp-resistance

3.1.4 Antibodies

3.1.4.1 Primary antibodies for indirect immunofluorescence

The following primary antibodies diluted in 0,5 % (w/v) λ-Carrageenan were used for immunocyto- and immunohistochemistry.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Species</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>1:100</td>
<td>mouse</td>
<td>Developmental Studies Hybridoma Bank (Iowa, USA)</td>
</tr>
<tr>
<td>CNPase</td>
<td>1:1000</td>
<td>mouse</td>
<td>Sigma</td>
</tr>
<tr>
<td>NeuN</td>
<td>1:1000</td>
<td>mouse</td>
<td>Chemicon (Temecula, CA, USA)</td>
</tr>
<tr>
<td>Synaptic vesicle protein-2 (SV-2)</td>
<td>1:100</td>
<td>mouse</td>
<td>Developmental Studies Hybridoma Bank</td>
</tr>
<tr>
<td>βIII-Tubulin</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Hiss Diagnostics (Freiburg)</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>1:2000</td>
<td>rabbit</td>
<td>R&amp;D Systems (Minneapolis, USA)</td>
</tr>
<tr>
<td>GFAP</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Dako (Hamburg)</td>
</tr>
<tr>
<td>Glutamic acid decarboxylase (GAD 65/67)</td>
<td>1:500</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>1:100</td>
<td>rabbit</td>
<td>Chemicon</td>
</tr>
<tr>
<td>BrdU</td>
<td>1:200</td>
<td>rat</td>
<td>Abcam (Cambridge, UK)</td>
</tr>
<tr>
<td>Choline acetyltransferase (ChAT)</td>
<td>1:100</td>
<td>goat</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Doublecortin</td>
<td>1:100</td>
<td>goat</td>
<td>Santa Cruz Biotechnologies (Santa Cruz, USA)</td>
</tr>
<tr>
<td>GFP</td>
<td>1:2500</td>
<td>chicken</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

**Table 1:** Primary antibodies used for indirect immunofluorescence.
3.1.4.2 Primary antibodies for Western blot analysis

Primary antibodies used for Western blotting were diluted in blocking buffer.

Table 2: Primary antibodies used for Western blot analysis.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Species</th>
<th>Reference/Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenascin-R</td>
<td>1:10</td>
<td>mouse</td>
<td>(Pesheva et al., 1989)</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>1:5000</td>
<td>rabbit</td>
<td>Covance (Berkeley, USA)</td>
</tr>
</tbody>
</table>

3.1.4.3 Secondary antibodies

For indirect immunofluorescence and Western blot analysis, Cy2™, Cy3™, and Cy5™ conjugated antibodies (diluted 1:200) and horseradish peroxidase-conjugated antibodies (diluted 1:10,000) to mouse, rabbit, rat, goat or chicken IgG were used, respectively (all from Dianova, Hamburg, Germany).

3.1.5 RNA and DNA preparation kits

BCA Kit (Pierce, Rockford, IL, USA)
Mini Preparation Kit (Amersham Pharmacia; Freiburg, Germany)
Maxi Preparation Kit (Qiagen; Hilden, Germany)
PCR Purification Kit (Qiagen)
Qiaquick Gel Extraction Kit (Qiagen)
Rapid Purification Kit (Qiagen)
RNase-free DNase Set (Qiagen)
RNeasy Mini Kit (Qiagen)
SuperScript First Strand Synthesis System (Invitrogen)
3.1.6 Bacteria and eukaryotic cells

*Escherichia coli* DH5α Invitrogen

**CHO-K1**
Chinese Hamster Ovary cell line K1

**MEF cells**
Mouse embryonic fibroblasts isolated from C57black/6J mice at embryonic day 14 (E.14)

**GFP⁺ ES cells**
Embryonic stem cell line derived from transgenic C57 black/6J mice expressing GFP under the influence of the chicken β-actin promoter (Okabe et al., 1997)

3.1.7 Bacterial media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Concentration</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-medium</td>
<td>10 g/l</td>
<td>Bacto-tryptone, pH 7.4</td>
</tr>
<tr>
<td></td>
<td>10 g/l</td>
<td>NaCl</td>
</tr>
<tr>
<td></td>
<td>5 g/l</td>
<td>Yeast extract</td>
</tr>
<tr>
<td>LB/Amp-medium</td>
<td>100 mg/l</td>
<td>Ampicillin in LB-medium</td>
</tr>
<tr>
<td>LB/Amp-plates</td>
<td>15 g/l</td>
<td>Agar in LB-medium</td>
</tr>
<tr>
<td></td>
<td>100 mg/l</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>LB/Cmp-medium</td>
<td>50 mg/l</td>
<td>Chloramphenicol in LB-medium</td>
</tr>
<tr>
<td>LB/Cmp-plates</td>
<td>15 g/l</td>
<td>Agar in LB-medium</td>
</tr>
<tr>
<td></td>
<td>50 mg/l</td>
<td>Chloramphenicol</td>
</tr>
</tbody>
</table>

Ingredients were dissolved in deionized water. Media were autoclaved and antibiotics were supplemented prior to use.
3.1.8 Cell culture media

Media were purchased from Invitrogen and supplemented as described

**CHO-cell medium**
- Dulbecco’s MEM (DMEM) supplemented with
  - 10% (v/v) Fetal calf serum (FCS)
  - 50 U/ml Penicilline/Streptomycine
  - 1 mM Sodium pyruvate
  - 2 mM L-Glutamine

**ES-medium**
- Dulbecco's MEM (DMEM, 4.5 g/l D-glucose, 25mM HEPES, 0.58 g/l L-glutamine, no sodium pyruvate) supplemented with
  - 15% (v/v) Fetal calf serum (FCS, ES quality, (PAA, Cölbe, Germany)
  - 2 mM L-glutamine
  - 1 × MEM non-essential amino acids
  - 1 mM Sodium pyruvate
  - 1 × Nucleoside mix (containing 3mM of each adenosine, cytidine, guanosine, uridine and thymidine in PBS)
  - 0.1 mM 2-mercaptoethanol
  - 50 U/ml Penicillin
  - 1,000 U/ml Murine leukemia inhibitory factor (ESGRO™ LIF)
    (Chemicon, Temecula, CA, USA)

**ES selection-medium**
- ES-medium supplemented with
  - 250 µg/ml G418 (Geneticin; Invitrogen)

**MEF-medium**
- DMEM (4.5 g/l D-glucose, with Glutamax™ I, no pyruvate) (Invitrogen) supplemented with
  - 9% FCS
  - 1 × MEM non-essential amino acids
  - 50 U/ml Penicillin
Stage 3-medium: DMEM/Ham’s F12 (1:1) supplemented with:
- \(1 \times\) N2-Supplement (Invitrogen)
- 5 \(\mu\)g/ml Fibronectin (Harbor Bio-Products, Norwood, Ma, USA)
- 2 mM L-glutamine
- 50 U/ml Penicillin

Stage 4-medium: DMEM/F12 supplemented with:
- \(1 \times\) B27-supplement (Invitrogen)
- 2 mM L-glutamine
- 50 U/ml Penicillin
- 20 ng/ml basic fibroblast growth factor (bFGF; FGF-2) (PreproTech, Rocky Hill, NY, USA)

Stage 5-medium (mN3) is Stage 4-medium without FGF-2

### 3.2 Methods

#### 3.2.1 Molecular biology methods

If not indicated otherwise, standard molecular biological techniques were carried out as described (Sambrook, Fritsch, and Maniatis, 1989).

#### 3.2.1.1 RNA isolation from cultured cells

Total RNA was isolated from undifferentiated and differentiated ESCs using the RNeasy Mini Kit (Qiagen) in combination with the RNase-free DNase Set (Qiagen) in order to get rid of genomic DNA during RNA extraction.

Undifferentiated and differentiated cells were washed three times with PBS and lysed with 500 \(\mu\)l RLT-lysis buffer containing 1% \(\beta\)-mercaptethanol. 500 \(\mu\)l 70% ethanol were added to the lysate and the suspension was transferred to an extraction column and centrifuged at 10,000 rpm for 15 sec. The filtrate was discarded and 350 \(\mu\)l of RW1 buffer was added to the column. After spinning 80 \(\mu\)l DNAse solution were given onto the column for 20 min.
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Afterwards, 350 µl of RW1 buffer was added to the DNAse solution. After spinning, the column was washed twice with 500 µl RPE buffer and RNA was eluted with 50 µl RNase-free water by centrifugation for 1 min at 10,000 rpm. RNA-concentrations were determined spectrosopically using an Amersham-Pharmacia spectrometer.

3.2.1.2 Reverse transcription of cDNA fragments

Messenger RNA was reverse transcribed into cDNA using the SuperScript First Strand Synthesis System (Invitrogen). Briefly, 500 ng oligo(dT)$_{12-18}$-primer (Invitrogen) were added to 500 ng RNA and the solution was filled up with RNase-free water to a total volume of 12 µl. The solution was incubated for 10 min at 70°C and put on ice afterwards. Thereafter, 4 µl „First Strand“-buffer, 2 µl 0,1M DTT and 1 µl 10mM dNTPs were added and the solution was heated to 42°C for 2 min before adding 1µl of Superscript™II-polymerase. Reverse transcription of RNA was carried out for 50 min at 42°C and the reaction was stopped by incubating the solution for 5 min at 95°C.

3.2.1.3 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a MWG-PCR thermocycler (MWG, Biotech, Ebersberg). Templates included transgenic DNA and cDNA. DNA was prepared from CHO and undifferentiated ESCs by incubating cells with 100 µl Boston buffer at 55°C over night. Amplification of transgenic DNA and cDNA fragments was carried out with *Taq*-Polymerase (Invitrogen). The following reaction mixture was used:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>2-10 ng</td>
</tr>
<tr>
<td>Primer A (10pM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer B (10pM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nucleotides (dNTPs) (20 mM)</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR-buffer (10 x)</td>
<td>5 µl</td>
</tr>
<tr>
<td>MgCl$_2$ (50mM)</td>
<td>1,5 µl</td>
</tr>
<tr>
<td>DNA-Polymerase</td>
<td>2.5 U</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>ad 50 µl</td>
</tr>
</tbody>
</table>

PCR was performed with primers purchased from Metabion (Martinsried, Germany). Initially, the reaction solutions were incubated for 2 min at 95°C. Afterwards, cycles of denaturation at
95°C for 40 sec, annealing of primers for 40 sec and elongation of fragments for different time periods led to specific amplification of DNA fragments. The conditions are listed in the following table.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers in 5’ - 3’- orientation</th>
<th>Length of the amplified fragment (bp)</th>
<th>'Annealing'-temperature (°C)</th>
<th>Time of elongation (sec)</th>
<th>Number of cycles</th>
</tr>
</thead>
</table>
| transgenic TNR | - GAT AGT GGA TGC TGA GGA CAC CT -  
- CAG ATC CTC TTC TGA GAT GAG TTT T -  
(myc-end) | 858 | 60 | 90 | 32 |
| β-tubulin | - GGA ACA TAG CCG TAA ACT GC -  
- TCA CTG TGC CTG AAC TTA CC - | 317 | 60 | 50 | 32 |

**Table 3:** Primers and conditions for semiquantitative PCR.

### 3.2.1.4 Gelelectrophoresis

Horizontal electrophoretic separation of DNA fragments was carried out in electrophoresis chambers (BioRad) using agarose gels. Gels were prepared by heating 1-2 % (w/v) agarose (Invitrogen) in 1× TAE buffer, depending on the size of DNA fragments. The gel was put in the electrophoresis chamber and covered with 1× TAE buffer. DNA sample buffer was added to the DNA and samples were pipetted in the sample pockets. The gel was run at constant voltage (10V/cm gel length) for 45-60 min. Afterwards, the gel was stained in an ethidiumbromide (Serva) staining solution for 20 min. Finally, gels were documented using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany).

### 3.2.1.5 Production and maintenance of competent bacteria

DH5α bacteria were streaked on LB-plates and grown overnight at 37°C. 50 ml of LB-medium was inoculated with 5 colonies and grown at 37°C until the culture had reached an optical density (OD600) of 0.3-0.5. Bacteria were stored as glycerol stocks (LB-medium, 25% (v/v) glycerol) at -80°C or were immediately used for further experiments.
3.2.1.6 Transformation of bacteria

For genetic modification of bacteria, either 50-100 ng of plasmid DNA or 20 µl of ligation mixture were added to 100 µl of competent DH5α bacteria followed by an incubation for 30 min on ice. After a heat shock (42°C, 2 min) and an incubation on ice (3 min), 800 µl of LB-medium were added to the bacteria and incubated at 37°C for 30 min. Cells were then centrifuged (10,000 × g) for 1 min at room temperature (RT) and the supernatant was removed. Cells were resuspended in 100 µl LB medium and plated on LB plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight and stored at 4°C up to 6 weeks afterwards.

3.2.1.7 Plasmid isolation from transformed bacteria

3.2.1.7.1 Plasmid isolation from 3 ml cultures (Minipreps)

Single bacterial colonies were isolated with a sterile pipette tip from plates and were separately incubated in 3 ml LB/Amp-Medium (100 µg/ml ampicillin) over night at 37°C with constant agitation. Cultures were transferred into 2 ml Eppendorf tubes and cells were pelleted by centrifugation (12,000 rpm, 1min, RT). Plasmids were isolated from the bacteria using the Amersham Pharmacia Mini Preparation Kit according to the manufacturer’s protocol. The DNA was eluted from the columns with 50 µl Tris-HCl (10 mM, pH 8.0).

3.2.1.7.2 Plasmid isolation from 200 ml-cultures (Maxipreps)

Large quantities of DNA were prepared using the Qiagen Maxiprep Kit. A single colony was added to 200ml LB/amp medium (100 µg/ml ampicillin) and the culture was incubated at 37°C with constant agitation overnight. Cells were pelleted in a Beckmann centrifuge (6,000g, 15 min, 4°C) and DNA was isolated as described in the manufacturer’s protocol. Finally, the DNA pellet was resuspended in 150 µl of pre-warmed (70°C) Tris-HCl (10 mM, pH 8.0) and the DNA concentration was determined.

3.2.1.8 Enzymatic digestion of DNA

Restriction of DNA was performed by incubating the DNA with twice the recommended amount of appropriate enzymes in the recommended buffer in the presence of BSA for 2 h. Enzymatic digestion was terminated by addition of sample buffer. If two enzymes were incompatible with each other, the DNA was digested successively with the enzymes. The
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DNA was purified between the two digestion steps using the Rapid Purification Kit (Qiagen). For analysis, DNA fragments were separated by electrophoresis in agarose gels.

3.2.1.9 Polishing of sticky ends
Blunting of non-compatible sticky ends was performed with Klenow enzyme. After purification of the DNA fragments, 125 µM dNTPs and Klenow buffer were added to the reaction volume. The Klenow enzyme was added (1 U, 30 min, RT) and the reaction was terminated by incubation at 70°C for 10 min. The fragments were used for ligation without further purification.

3.2.1.10 Ligation of DNA-fragments
For ligation of DNA fragments, 50 ng vector DNA were mixed with the three- to fivefold molar excess of insert DNA. 1 µl of T4-Ligase and 1 µl of ligation buffer were added and the reaction mix was filled up to a final volume of 10 µl. The reaction was incubated either for 2 h at room temperature or overnight at 16°C. The reaction mixture was used directly for transformation without any further purification.

3.2.1.11 Extraction of DNA fragments from agarose gels
For extraction of DNA fragments from agarose gels, ethidiumbromide-stained gels were illuminated with UV-light and the appropriate DNA band was excised from the gel with a clean scalpel. The gel fragment was transferred into an Eppendorf tube and DNA was isolated using the Qiaquick Gel Extraction Kit according to the manufacturer’s instructions. The DNA fragment was eluted with 50 µl pre-warmed (70°C) Tris-HCl (10 mM, pH 8.0) and the DNA-concentration was determined using the undiluted eluate.

3.2.1.12 Purification of DNA fragments
DNA fragments were purified using the Rapid PCR Purification Kit (Qiagen). Purification was performed according to the manufacturer’s protocol. The DNA was eluted from the column by addition of 50 µl pre-warmed (70°C) Tris-HCl (10 mM, pH 8.0) and the DNA-concentration was determined using the undiluted eluate.
3.2.1.13 Determination of DNA concentrations

The concentration of DNA was determined spectroscopically using an Amersham-Pharmacia spectrometer. The DNA was diluted 1:50 with water and the solution was transferred into a 50 µl cuvette. The concentration was determined by measuring the absorbance at 260 nm, 280 nm and 320 nm. Absorbance at 260 nm had to be higher than 0.1 but less than 0.6 for reliable determinations. A ratio of A$_{260}$/A$_{280}$ between 1.8 and 2 monitored a sufficient purity of the DNA preparation.

3.2.1.14 Phenol/chloroform extraction of DNA

For extraction of DNA, an equal volume of phenol/chloroform solution (1:1) was added to the aqueous, DNA-containing sample and vortexed for 1 - 2 min creating an emulsion. After centrifugation at 14,000 $\times$ g for 2 - 5 min at RT, the aqueous/top layer was carefully transferred into a new tube. An equal volume of TE buffer was added to the lower/organic phase and the remaining DNA was extracted by centrifugation and transfer of the upper phase to the same tube. In order to remove residual phenol, the combined aqueous phases were extracted with an equal volume of chloroform. After centrifugation at 14,000 $\times$ g for 5 min at RT, the aqueous/top layer containing the DNA was carefully transferred to a new tube.

3.2.1.15 Precipitation of DNA

The aqueous DNA solution was mixed with 1/10 volume of sodium acetate, pH 5.2. Afterwards, 2,5 volumes of cold ethanol (-20°C) were added and the samples were mixed well. Following incubation on ice for 30 min, samples were centrifuged for 10 - 15 min (14,000 $\times$ g, RT). Supernatants were carefully removed and 1 ml cold 75% (v/v) ethanol (-20°C) was added to each sample. Tubes were inverted several times and centrifuged for 5 min (14,000 $\times$ g, RT). For optimal purity, the pellet was loosened from the tube during inverting and was broken up in ethanol. The washing step was repeated twice. Supernatants were removed and DNA pellets were air dried (approximately 5 min at RT). DNA was dissolved in 50µl pre-warmed Tris-HCl (5 mM, pH 8.0).

3.2.1.16 DNA Sequencing

Sanger-DNA-sequencing was performed by the sequencing facility of the Center for Molecular Neurobiology Hamburg (ZMNH). For preparation, 1 µg of plasmid DNA was
diluted in 7 µl sterile deionized water and 1 µl of the appropriate sequencing primer (5 pM) was added. The oligonucleotides are listed in the Appendix.

3.2.2 Protein-biochemical methods

3.2.2.1 Removal of N-glycans from proteins

For removal of N-glycosidically-linked carbohydrate residues from proteins, 16,5 µl medium supernatant were mixed with 2 µl 10x phosphate buffer (pH 6.9), 1µl NP-40 and 0,5 µl PNGase F (Roche, Mannheim). The solution was incubated over night at 37°C.

3.2.2.2 Preparation of protein solutions from cells

Cells were grown to confluency and washed three times with PBS. Cells were lysed with 100 µl Ripa buffer at 4°C for 45 min. Cells were scraped off from the surface and transferred into 1,5 ml Eppendorf tubes. For analysis of expression in vivo, GFP+ cells were isolated from brains of homozygous TNR−/− mice under a fluorescence microscope (Axioplan 2; Carl Zeiss Microimaging, Thornwood, NY). Cells were incubated with 100µ Ripa buffer for 45 min at 4°C. Cell lysates were stored at -20°C.

3.2.2.3 Determination of protein concentration

The BCA Kit (Pierce) was used to determine the protein concentration of cell lysates. Solution A and B were mixed in a ratio of 1:50. Afterwards, 200 µl of the resulting BCA solution were added to 10 µl of the cell lysate in microtiter plates and the mixture was incubated for 30 min at 37°C. A BSA standard curve was co-incubated ranging from 50 µg/ml to 1 mg/ml. The extinction of the samples was determined at 568 nm in a microtiter plate reader.

For qualitative and quantitative evaluation of proteins in the medium supernatant, the amount of medium supernatant taken for Western blot analysis was normalized to the number of cultured cells.

3.2.2.4 SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) using the Mini-Protean III
system (BioRad, Hercules, CA, USA). The running and the stacking gel had the following composition.

<table>
<thead>
<tr>
<th></th>
<th>8 % Running gel</th>
<th>4 % Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Acrylamide / 0,8 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N’N’-Methylen-bisacrylamide (Serva)</td>
<td>1,6 ml</td>
<td>400 µl</td>
</tr>
<tr>
<td>Deionized water</td>
<td>2,1 ml</td>
<td>1,6 ml</td>
</tr>
<tr>
<td>1,5 M Tris (pH 8,8; Biomol)</td>
<td>2,3 ml</td>
<td>-</td>
</tr>
<tr>
<td>0,5 M Tris (pH 6,8; Biomol)</td>
<td>-</td>
<td>300 µl</td>
</tr>
<tr>
<td>10 % SDS-solution (Serva)</td>
<td>60 µl</td>
<td>30 µl</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED; Merck)</td>
<td>6 µl</td>
<td>6 µl</td>
</tr>
<tr>
<td>10 % Ammoniumpersulphate (APS; Biorad)</td>
<td>15 µl</td>
<td>15 µl</td>
</tr>
</tbody>
</table>

**Table 4:** Composition of running and stacking gel.

After complete polymerization of the gel, the chamber was assembled as described by the manufacturer’s protocol. Samples were mixed with 5x sample buffer and incubated for 5 min at 95°C. Up to 25 µl sample were loaded in the pockets and the gel was run at constant 120 V for 10 min and then at 180V for 45 - 60 min. Gels were subsequently subjected to Western blotting.

### 3.2.2.5 Western blot analysis

#### 3.2.2.5.1 Electrophoretic transfer of proteins

The transfer of proteins onto a nitrocellulose membrane was performed according to a method described by Towbin et al. (1979). Proteins separated by electrophoresis were transferred from the SDS-gel on a membrane (Protran Nitrocellulose BA 85, 0,45 mm, Schleicher & Schüll, Dassel, Germany) using a MINI TRANSBLOT-apparatus (BioRad). The gel was equilibrated in blot buffer for 5 min before the blotting sandwich was assembled as described in the manufacturer’s protocol. Proteins were transferred by electrophoresis at 4°C in blot buffer at 80 V for 120 min. The pre-stained marker BenchMark™ (Invitrogen) was used as a molecular weight marker.
3.2.2.5.2 Detection of proteins on nitrocellulose membranes

After blotting, the membrane was removed from the sandwiches and incubated with 10 ml blocking solution for 1 hour at RT. Afterwards, the primary antibody was added in the appropriate dilution over night at 4°C. The primary antibody was collected and the membrane was washed six times with TBST for 10 min in order to remove remaining unbound antibodies. The appropriate secondary antibody was applied for 1 h at RT. The membrane was washed again six times for 10 min with TBST. Antibody-labeled proteins were visualized using the enhanced chemiluminescence detection system (Pierce). The membrane was soaked for 1 min in detection solution (1:1 mixture of solutions I and II) and was then placed between two cling films. The membrane was exposed to X-ray films (Biomax-MR, Eastman Kodak, Rochester, NY, USA) for several time periods, starting with a 2 min exposure. Films were developed according to standard procedures.

3.2.3 Cell culture

3.2.3.1 General techniques

Cells were propagated and differentiated in an incubator (Nuaire, Plymouth, Maine, USA) with 5 % CO₂ at 37°C. Cells were handled under sterile conditions using a cell culture bench with laminar flow (SterilGard Class II, The Baker Company, Sanford, Maine, USA).

3.2.3.1.1 Surface coating

For culture of mitomycin C-inactivated mouse embryonic fibroblasts, culture dishes were covered with 0,1 % gelatine for 30 min. Afterwards, the gelatine was removed and dishes were air dried. For propagation and differentiation of ESC-derived cells, cell culture dishes and coverslips were incubated with 15 % poly-L-ornithine for 3 hours at 37°C. Thereafter, dishes were washed with PBS three times before applying cells.

3.2.3.1.2 Enzymatic dissociation of cells

Cells were washed three times with PBS. Embryonic fibroblasts and differentiating ESCs were incubated with 0,05 % trypsin/EDTA solution and undifferentiated ESCs were incubated with 0,25 % trypsin/EDTA solution for 5 min at 37°C. CHO cells were covered with Versene (Invitrogen) for 5 min at 37°C. Cells were dissociated with a fire-polished glass pipette and the
reaction was stopped by adding a threefold volume of serum-containing medium to the cells. Centrifugation of cells was performed at 1.000 rpm for 5 min and cells were resuspended in fresh medium.

3.2.3.1.3 Freezing of cells

Cells were trypsinized, washed with PBS and resuspended in 1 ml freezing-medium that contained 50 % FCS, 40 % cell culture medium and 10% (v/v) DMSO. Cells were transferred to freezing tubes and cooled down to -80°C within 24 hours. Finally, tubes were transferred to liquid nitrogen tanks (-196°C) for long term storage.

3.2.3.1.4 Thawing of cells

Tubes with frozen cells were warmed up in a waterbath pre-heated to 37°C. Thawed cells were immediately diluted in 9 ml culture medium and centrifuged for 5 min at 1.000 rpm. Afterwards, cells were resuspended in appropriate medium and volume.

3.2.3.1.5 Counting of cells

Cells were resuspended in 10 ml culture medium and 20 µl were transferred to a Neubauer counting chamber. Cells were counted in four counting squares and the concentration of cells was evaluated using the following formula:

\[ \text{Number of cells / ml} = \frac{\text{cells counted} \times 10.000}{4} \]

3.2.3.2 Culture and transfection of CHO cells

CHO-K1 cells were cultured in 75 cm² flasks with 10 ml culture medium containing 10 % FCS. For propagation, CHO cells were passaged 1:10 on new 75 cm² flasks when grown to confluency.

For transfection of cells, 2 x 10^5 CHO cells were plated on six-well plates (Greiner) and were cultured for 24 hours, thereby reaching a density of 80-90 %. The Lipofectamine Plus Kit (Invitrogen) was used for transfection of cells. Following the manufacturer’s instructions, cells were washed with medium that did not contain FCS and antibiotics and were transfected with 2 µg plasmid-DNA per well. 6 µl Plus Reagent and 4 µl Lipofectamine were used per well. The transfection was terminated after 3 h by addition of an equal volume of DMEM.
supplemented with 20 % FCS and 2% antibiotics (P/S). 24 h after transfection, cells and the medium supernatant were collected for Western blot analysis.

**3.2.3.3 Culture of embryonic stem cells (ESCs)**

**3.2.3.3.1 Inactivation of mouse embryonic fibroblasts**

The propagation of undifferentiated ESCs requires co-cultivation with mouse embryonic fibroblasts (MEFs) that secrete factors including leukaemia inhibitory factor (LIF). Neomycin-resistant MEFs were derived from E13.5 - E14.5 embryos of neo-transgenic mice (FV-Neo, kindly provided by Dr. Michael Boesl, ZMNH, Hamburg). These fibroblasts were propagated and afterwards mitotically inactivated with the chemotherapeutic agent mitomycin C (MMC, Roth, Karlsruhe, Germany). Confluent cultures of neomycin-resistant fibroblasts were incubated 2 - 3 hours with MEF-medium containing 10 mg/ml mitomycin C. After washing three times with PBS, cells were trypsinized, seeded on gelatine-coated culture dishes with a density of $5 \times 10^4$ cells per cm$^2$ and were cultured in MEF-medium for 24 hours before applying ESCs to these cells. Alternatively, MMC-inactivated MEFs were stored as aliquots in liquid nitrogen.

**3.2.3.3.2 Propagation of undifferentiated ESCs**

For this study, an ES cell line was used that constitutively expresses enhanced green fluorescent protein (eGFP) under the control of the chicken $\beta$-actin promoter (Abbondanzo et al., 1993) derived from transgenic C57BL/6J mice ubiquitously expressing eGFP (Okabe et al., 1997). GFP$^+$ ES cells were propagated on MMC-inactivated MEFs in ES-medium and formed stem cells colonies. In order to avoid differentiation of ES cells, ES-medium was changed every day and ES cells were trypsinized 1:2 to 1:5 every 24 - 72 hours.

**3.2.3.3 Electroporation and clonal analysis of ESCs**

GFP$^+$ ES cells were electroporated with the TNR-expression construct p901-PGK-neo-PGK-TNRmyc or with the empty vector p901-PGK-neo to generate TNR-overexpressing (TNR$^+$) or mock-transfected (TNR-) ES cell clones, respectively (cloning described in chapter 4.1 and Appendix). The TNR-expression vector and the control vector were linearized with SalI and subsequently purified by gel extraction, phenol-chloroform extraction and ethanol precipitation. Using a Gene Pulser system (Bio-Rad), the linearized plasmid-DNA was
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electroporated into 10⁷ GFP⁺ ES cells diluted in 800 µl PBS at 0.25 kV and 500 µF. Cells were incubated at RT for 5 min and plated on six 10 cm diameter dishes containing inactivated G418/neomycin-resistant feeder cells (see above). Following 24 hours of recovery in ES-medium, the medium was exchanged against the same medium containing 250 µg/ml active G418. Transfected cells were kept in culture for 7 days and the selection medium was changed every day. Colonies reached a macroscopic size sufficient for picking them with pipette tips.

168 G418-resistant clones that had been electroporated with the p901-PGK-neo-PGK-TNRmyc vector and 24 clones of the electroporation with the p901-PGK-neo plasmid were picked and trypsinized in 50 µl 0.25% trypsin-EDTA at 37°C for 5 min. Cells were dissociated and individually replated in 96-well dishes with feeder cells. After seeding, cells were allowed to re-attach and grown to reach almost confluency. Again, cells were subcultivated (1:2 to 1:5). Almost confluenly grown 96-well-plates were subjected to PCR-screening using primers, one binding to the 3’ end of TNR and the other binding to the myc tag sequence, thus detecting transgenic TNR-DNA (described in chapter 3.2.1.3). The medium supernatant of these clones was analyzed for TNR-protein expression with Western blotting. For cryo-preservation, cells were trypsinized as described above. An equal amount (100 µl) of concentrated freeze medium (80% ES-qualified FCS, 20% (v/v) DMSO) was added and wells were overlayed with 75 µl mineral oil. For storage of ES cell clones, plates were slowly frozen to - 80°C. For expansion of individual ES cell clones, the corresponding 96-well plate was quickly thawed at 37°C and clones were replated in 24-well plates. Subsequently, ES cells were subcultivated and seeded in 6-well, 6 cm and 10 cm culture dishes. At all stages, aliquots of cells were frozen and cryo-preserved in liquid nitrogen.

3.2.3.3.4 Differentiation of ESCs

For differentiation of ES cells into postmitotic neural cells the lineage selection protocol according to Okabe et al. (1996) and Lee et al. (2000) was used in a slightly modified form. This protocol covers 5 stages and starts with the propagation of undifferentiated murine ES cells on a monolayer of MMC-inactivated mouse embryonic fibroblasts in ES-medium containing 1.000 U/ml LIF (stage 1; Fig. 2).
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**Figure 2:** *In vitro*-differentiation of murine ES cells into postmitotic neural cells.

As schematically shown, undifferentiated ES cells were propagated in stage 1 and differentiation was induced by the formation of embryoid bodies via hanging drops (stage 2). Afterwards cells were selected for nestin-positives neural precursor cells (stage 3) and selected cells were propagated to a sufficient number up to 4 weeks in stage 4. Differentiation into neurons, astrocytes and oligodendrocytes was induced by withdrawal of FGF-2 (stage 5).

After propagation, differentiation of ES cells was induced by the formation of embryoid bodies (EBs; stage 2). For that, ES cells were trypsinized and 2 million cells were diluted in 50 ml ES-medium without LIF. About 2,500 drops of 20 µl volume (800 cells) were plated in the inner surface of Petri dish lids. Lids were carefully turned upside down and placed on the bottom of the dishes that contained 8 ml PBS. Differentiating cells in hanging drops aggregated due to gravity and formed EBs during 2 days. Afterwards, EBs in hanging drops were washed off and cultured in bacterial Petri dishes for additional 2 days in ES-medium without LIF. Thereafter, EBs were transferred to adherent cell culture dishes and were cultured for 1 week in serum-free stage 3 medium containing 1 % N2-supplement and 5 µg/ml fibronectin in order to select for nestin-positive neural precursors cells (stage 3).

Selected neural precursors were expanded on PLO-coated tissue culture dishes for 14 - 28 days (stage 4) in medium consisting of DMEM/F12 medium supplemented with 2 % B-27, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 20 ng/ml FGF-2 (mN3-bFGF). The medium applied to the cells in stage 4 differed from the medium mN3FL described by Lee et al. (2000) by the omittance of PDGF and laminin, and by replacement of insulin, transferrin, selenium, progesterone, hydrocortisone, and putrescine by B-27 supplement. Differentiation into postmitotic neural cells was induced by withdrawing the FGF-2 mitogen for 3 to 14 days depending on the experimental design (stage 5).
3.2.4 Immunocytochemistry

3.2.4.1 Immunocytochemistry of cultured cells

Cells were grown on plastic coverslips (Sarstedt, Numbrecht, Germany). The medium supernatant of cultured cells was removed and cells were washed three times for 5 min with PBS (pH 7.3). After fixation for 20 minutes in 4% paraformaldehyde, cells were incubated with blocking solution for 30 min. Primary antibodies in appropriate dilutions were applied overnight at 4°C. Afterwards, cells were washed with PBS for 5 min, 8 min and for additional 8 min at RT before appropriate secondary antibodies were applied for 60 minutes at RT. After one washing step with PBS, cells were incubated with 50 µg/ml DAPI (Sigma) for 10 min to stain cell nuclei. Finally, coverslips were washed three times with PBS and once with deionized water before mounted on glass slides with Aqua Poly-Mount medium (Polysciences, Warrington, PA, USA). Cells were examined with a fluorescence microscope (Axioplan 2; Carl Zeiss Microimaging, Thornwood, NY, USA). The degree of differentiation was determined by calculating the ratio of the total number of cell type-specific marker-positive cells among all GFP+ cells per visual field at 40 x magnification.

3.2.4.2 BrdU-labeling in vitro

For labeling with bromodeoxyuridine (BrdU) in vitro, BrdU was added to the culture medium to a final concentration of 10 mM eight hours before fixation. Afterwards, cells were washed with PBS, dehydrated with 70%, 95%, 100%, 95% and 70% ethanol for 2 min for each step and were then incubated in 2M HCl at 37°C for 30 min. Cells were washed 3 times with PBS before applying the blocking solution and the primary antibody overnight at 4°C.

3.2.5 Scratch assay

TNR+ or TNR- cells were differentiated to stage 4 and grown to confluency. Using a sterile pipette tip, a cell-free space of about 2 mm was produced by performing a single scratch through the monolayer of cells. Scratched cells were removed by washing culture dishes three times with PBS. Cells were then cultured for 48 hours in the absence of FGF-2 and then fixed in 4% paraformaldehyde in PBS. The number of GFP+ cells was measured per visual field (40x magnification) in scratched and non-scratched areas. Experiments were performed with mixed cultures ranging from 100% TNR+ cells to 75% TNR+ cells (and 25% TNR- cells),

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50% TNR⁺ cells (and 50% TNR⁻ cells), 25% TNR⁺ cells (and 75% TNR⁻ cells) and 100% TNR⁻ cells.

**3.2.6 Transplantation of cells and evaluation of grafts**

**3.2.6.1 Transplantation of pre-differentiated stem cells**

The right striatum of 2-month-old C57BL/6J male mice and of 10-month-old TNR⁻/- male mice was lesioned by stereotaxic injection of 1 µl of 60 nmol quinolinic acid (Sigma) at the following coordinates in relation to bregma: 0.4 mm anteroposterior, 2.4 mm mediolateral, and 3.6 mm dorsoventral. ES cells were differentiated for 3 days in stage 5, dissociated by 0.05% trypsin/0.04% EDTA and resuspended in cold PBS solution without Ca ++ and Mg ++ at a density of 100.000 viable cells per µl. Three days after lesioning, 1µl of the precursor cell suspension in PBS was stereotaxically infused into the right striatum at the same coordinates as for injection of quinolinic acid. Cells were carefully engrafted over a time period of 5 min and the injection pipette was left in the striatum for additional 3 min before slowly removing it from the brain. Sham-injected animals received a PBS injection instead of pre-differentiated stem cells.

The number of quinolinic acid-lesioned wild type mice studied was: TNR⁺ cells (n=17), TNR⁻ cells (n=18), and sham-injected controls (n=12). Six TNR⁻⁻ mice were lesioned and afterwards injected with TNR⁺ (n=3) or TNR⁻ cells (n=3). For analysis of BrdU incorporation mice were injected intraperitoneally every 12 hours with 100 mg BrdU/kg body weight (Sigma) from day 3 to 7 after grafting. Grafts were analyzed 1 and 2 months after transplantation. All animal experiments were approved by the University and State of Hamburg Animal Care Committees.

**3.2.6.2 Preparation of brain sections for histological analysis**

Mice were intracardially perfused with 4 % paraformaldehyde in 0,1M cacodylate buffer for 15 minutes. Two hours after perfusion brains were extracted and were post-fixed over night in the same solution. Afterwards, brains were cryoprotected with 15% sucrose for 24 to 48 hours at 4°C. Perfused brains were frozen in liquid nitrogen-cooled 2-methyl-butane (Sigma) and cut on a cryostat at 25 µm. Serial cryosections were mounted on SuperfrostPlus slides (Menzel, Braunschweig, Germany). Slides were stored at -20°C. For long term storage of
brains, perfused and cryoprotected brains were transferred to liquid nitrogen and stored for up to 12 months.

3.2.6.3 Immunohistochemistry of brain sections

Glass slides with fixed brain sections of 25 µm thickness were warmed to RT for 15 min and afterwards transferred to jars containing antigen retrieval solution pre-heated to 80°C. Slides were incubated for 30 min in antigen retrieval solution and afterwards cooled down to RT. Sections were blocked for 1 hour at RT with blocking solution and then incubated with primary antibodies in appropriate concentrations over night at 4°C. On the next day, slides were washed three times with PBS for 15 min and then incubated with appropriate secondary antibodies coupled to Cy2, Cy3, or Cy5 (all from Dianova, Hamburg, Germany) for 2 hours at RT. After washing the sections three times for 15 min at RT, slides were stained with bis-benzimide for 10 minutes and were washed twice for 5 min at RT afterwards. Finally, slides were mounted with Aqua Poly-Mount medium. Specimens were examined with the fluorescence or with a confocal laser-scanning microscope (LSM510; Carl Zeiss Microimaging). For analysis of differentiation, only cell type-specific marker-positive cells overlapping with the GFP signal were counted. The ratio of marker-positive cells was evaluated as described for the in vitro analysis.

3.2.6.4 Evaluation of the graft size and number of engrafted cells

A Neurolucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany) and an Axioskop microscope (Carl Zeiss) were used for quantitative analysis. Unbiased estimates of the volume of transplants and total number of grafted cells per animal were calculated 1 and 2 months after transplantation according to the optical disector and Cavalieri methods (Howard and Reed, 1998). The volume of the graft and the cell density within the graft were determined measuring every tenth 25 µm thick section of the graft. Transplanted cells were identified by their GFP signal. Graft areas were outlined on digitized images to calculate volumes considering section thickness and frequency. Using random sampling in the graft core and in the periphery of the graft, cell counts were performed according to the optical disector principle at a magnification of 40x. The total number of GFP⁺ neurons in the grafts was determined considering the percentage of GFP⁺ and NeuN⁺ or GFP⁺ and GAD65/67⁺ cells of all engrafted GFP⁺ cells and the total number of GFP⁺ cells within the grafts.
3.2.6.5 Measurement of distance migrated by engrafted cells

The graft edge was delineated at low magnification in digitized images. Shortest distances of at least 100 individual cells from the graft edge of recipient animals were determined at higher magnification (40x). Migration was measured at the medial, ventral and lateral aspect of the graft 1 and 2 months after transplantation of cells.

3.2.6.6 Analysis of migration of endogenous neuroblasts

The Neurolucida software-controlled computer system and an Axioskop microscope were used for counting host-derived neuroblasts migrating in the adult brain. Sections of 25µm thickness were immuno-stained with an antibody directed against doublecortin. The number of host-derived doublecortin+ cells was counted per section in the subventricular zone, in the posterior extension of the rostral migratory stream and in an area of 200µm x 200µm in the dorsal striatum between the rostral migratory stream and the GFP+ graft. Cell numbers were also counted in corresponding areas in the contralateral hemisphere.

3.2.7 Magnetic resonance imaging (MRI)

3.2.7.1 Labeling of differentiating cells

Differentiating TNR- ESCs were differentiated to stage 4. For labeling with iron particles, 100µl Resovist® containing 0.5mmol Fe/ml solution (Schering, Kenilworth, NJ, USA) were diluted in 10 ml stage 4 medium and cells were incubated with this solution for 24 hours. After labelling, cells were washed five times with PBS to remove free-floating Resovist® particles. Cells were grown for additional 48 hours, were washed five times with PBS and were then trypsinized. For transplantation, 100.000 labeled cells in 1 µl were infused in the right striatum of QA-injected mice three days after lesion using the same coordinates as described in 3.2.6.1.

3.2.7.2 Histochemical detection of Resovist®-labeled cells

In parallel, cells were fixed with 4% paraformaldehyde 72 hours after labeling with the Resovist®-solution. A Turnbull-staining (Romeis, 1989) was performed to mark incorporated iron particles. After fixation, cells were washed three times with PBS and incubated with a 10% ammoniumsulphide solution for 30 - 45 min at RT. Afterwards, cells were washed twice.
in deionized water and incubated with a ferricyanopotassium solution for 20 min at RT. Finally, cells were washed three times and mounted with Aqua Poly-Mount medium (Polysciences, Warrington, PA, USA).

3.2.7.3 Magnetic resonance imaging of QA-lesioned animals

QA-lesioned animals with or without grafts consisting of Resovist®-labeled cells were anaesthetized with 250 µl anaesthetic solution. The MRI scans were kindly performed by Dr. Kersten Peldschus of the Institute for Radiology, University Hamburg. Images were analyzed with Philips DICOM Viewer R1.1 software (Philips, Hamburg, Germany).

3.2.8 Behavioural analysis of mice

3.2.8.1 Rotarod test

Mice had to walk on a turning, corrugated rod (3.2 cm in diameter) (Accelerated Rotarod for mice, Jones & Roberts, TSE systems, Bad Homburg, Germany). Mice underwent 5 trials with an interval of 30 min. Trials 1 and 2 were performed at slow, constant speed (4 rpm) for a maximum duration of 3 min. Trials 3-5 were performed with the accelerating rod, starting with 4 rpm up to 40 rpm within 4 min, with a maximum duration of 5 min. The performance of the mice was evaluated by scoring the latency to fall.

3.2.8.2 Rotation test

For behavioural analysis 27 adult C57BL/6J mice received a unilateral striatal lesion through injection of 60nmol quinolinic acid. For assessment of functional lesion, a rotation bias was calculated as net ipsilateral turning angle divided by distance moved two days after lesion (1 day before transplantation). For this assay, animals were intraperitoneally injected with apomorphin (Sigma) at a concentration of 2 mg/kg body weight. Mice that did not rotate to the ipsilateral side were excluded from further investigation. Functionally lesioned mice were then randomly assigned to three groups, one for the transplantation of TNR⁺ cells (n=9), one for the transplantation of TNR⁻ cells (n= 10) and a third group which received only a PBS vehicle injection (sham-injected group, n=8). Apomorphin-induced rotation behaviour was tested 8, 21, 31 and 45 days after quinolinic acid treatment (5, 18, 28 and 42 days after transplantation of cells). The behavioral tests were performed at the beginning of the animals’
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dark phase cycle. Rotation was measured in an open field box for 30 minutes at 50 lux. Ethovision software (Noldus, Wageningen, The Netherlands) was used for processing data.

3.2.9 Statistical evaluation

All experiments were performed in a blinded manner and Student’s t-test was used for statistical evaluation, if not indicated otherwise.
4. Results

4.1 Generation of tenascin-R overexpressing murine embryonic stem cells

Murine embryonic stem cells were genetically modified to overexpress the extracellular matrix molecule tenascin-R. For that, an expression vector containing the full length cDNA of tenascin-R was electroporated into murine embryonic stem cells that were transgenic for the enhanced green fluorescent protein (eGFP⁺). The expression vector was first tested in chinese hamster ovary (CHO) cells in order to assess if this construct is expressed in eukaryotic cells.

4.1.1 Cloning of the tenascin-R expression vector

The full length cDNA of tenascin-R was amplified from adult mouse brain homogenate by PCR using sequence-specific primers and a proof reading DNA polymerase (fig. 3 A). Because of its length of around 4,2 kb two separate fragments of the tenascin-R cDNA were amplified and were ligated into the cloning vector pGEM-T-Easy. The sequence of the tenascin-R cDNA in pGEM-T-Easy was verified by DNA sequencing. Subsequently, the cDNA was cloned into a pcDNA3.1myc-his vector as a HindIII - NotI fragment to label the cDNA with a myc-tag at its 3’-end (fig. 3 B). In parallel, a 550 bp XhoI - HindIII fragment containing the promotor of the 3-phosphoglycerat-kinase (PGK) was cloned from a pBlueCam-PGK plasmid into the eukaryotic expression vector p901-PGK-neo that contained a PGK-promoter-driven neomycin resistance cassette (PGK-neo). This cloning step gave rise to a p901-PGK-neo-PGK construct (fig. 3 C). The PGK-promoter was chosen because it is known to be strongly expressed in murine embryonic stem cells. Finally, the myc tagged cDNA of tenascin-R was cloned into the p901-PGK-neo-PGK construct as a HindIII - HpaI/NaeI fragment generating the tenascin-R expression vector p901-PGK-neo-PGK-TNRmyc (fig. 3 D). Restriction analysis of this construct with XhoI and BamHI revealed expected fragments of 2,4 kb, 1,7 kb, 1,6 kb, 1,5 kb, 0,8 kb and 0,7 kb (fig. 3 F; see also Appendix). For negative control the p901-PGK-neo vector was used that did not contain the tenascin-R expression cassette (p901-PGK-neo).
Figure 3: Cloning of the tenascin-R expression vector.
(A) The full length cDNA of tenascin-R was amplified in two fragments from adult mouse brain and cloned into the pGEM-T-Easy cloning vector. (B) Afterwards, the full length cDNA of tenascin-R was inserted into the pcDNA3.1myc-his vector that contained a myc tag at the 3’ end of the multiple cloning site. (C) In parallel, a PGK-promotor driving the expression of the 3-phosphoglycerat-kinase was cloned into the eukaryotic expression vector p901-PGK-neo that contained a neomycin-phosphotransferase selection message also driven by a PGK-promotor. (D) The myc tagged tenascin-R-cassette was finally cloned into the p901-PGK-neo-PGK construct giving rise to the tenascin-R expression vector p901-PGK-neo-PGK-TNRmyc. The empty vector p901-PGK-neo vector served as negative control plasmid for transfection. (E) Legend. TNR, tenascin-R; PGK, 3-phosphoglycerat-kinase; neo, neomycin-phosphotransferase. (F) Restriction analysis of the p901-PGK-neo-PGK-TNRmyc construct with XhoI and BamHI revealed fragments of expected lengths.

4.1.2 Transfection of CHO cells
Eukaryotic CHO cells were transiently transfected by lipofection with the p901-PGK-neo-PGK-TNRmyc vector or with the p901-PGK-neo vector, respectively. The incorporation of the expression construct was assessed by PCR with one primer binding to the 3’ end of the tenascin-R cDNA and the other binding to the myc tag sequence (fig. 4 A). The expression of tenascin-R protein was determined 48 hours after transfection by Western blot analysis of the cell lysate and of the medium supernatant using the monoclonal tenascin-R-antibody “596”
(fig. 4 B). A strong band of 220 kDa was observed in p901-PGK-neo-PGK-TNRmyc vector transfected cultures (cell lysate and medium supernatant) in contrast to the negative control. This result shows that the incorporated construct was functional in a eukaryotic system leading to expression of the full length protein of tenascin-R.

Figure 4: Transfection of CHO cells with the tenascin-R expression vector. (A) PCR revealed that the transgenic construct could be amplified in CHO cells transfected with the tenascin-R expression vector (TNR⁺) using sequence specific primers. In contrast, in CHO cells transfected with the empty vector (TNR⁻) the specific band of 858 bp could not be observed. (B) Western blot analysis of the cell lysate (c.l.) and medium supernatant (m.s.) of TNR⁺ and TNR⁻ CHO cells showed that only TNR⁺ CHO cells expressed and secreted a tenascin-R protein with a molecular weight of 220 kDa. Positive control: CHO cells transfected with the pcDNA3.1TNRmyc plasmid.

4.1.3 Transfection of murine embryonic stem cells

The tenascin-R expression vector and the control vector were linearized and purified by phenol-chloroform extraction. Murine embryonic stem cells (ESCs) that expressed eGFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer were electroporated as described in Materials & Methods (chapter 3.2.3.3.3). Single stem cell colonies were isolated after propagation in the presence of G418. PCR-screening using the primers described above revealed that 123 clones out of 168 (73%) had incorporated the transgenic DNA encoding tenascin-R. As expected, none of the empty vector-transfected stem cell colonies showed the specific band of 858 bp (fig. 5 A). Western blot analysis of the medium supernatant collected from these 168 clones revealed that only 3 stem cell clones (1.7%) expressed and secreted the tenascin-R protein whereas none of the empty vector-
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transfected clones did (fig. 5 B). This specific expression was verified on RNA-level performing RT-PCR. Only these 3 clones expressed the transgenic tenascin-R construct whereas neo-transfected cells and stem cells that had incorporated the expression vector but where negative for tenascin-R on protein level did not (fig. 5 C). The strongest tenascin-R-expressing clone, termed 6B, was used for the following experiments. This clone will be abbreviated with TNR⁺. An empty vector-transfected ES cell clone (clone 3) was used for negative control and will be named TNR⁻.

Figure 5: Generation of tenascin-R overexpressing murine ESCs.
(A) PCR of transfected ESCs after clonal expansion using primers binding in the transgenic expression cassette. A representative distribution of clones is shown. The specific band of 858 bp (red arrow) became visible in about 73% of clones whereas negative control clones showed only an unspecific band of approximately 1100 bp as the majority of all clones did (red star).
(B) Western blot and (C) RT-PCR-analysis revealed that 3 clones expressed the transgenic tenascin-R construct and secreted a tenascin-R protein into the medium supernatant. TNR⁻ ESC clone 28a was a clone that had incorporated the expression construct and was positive on genomic PCR level but appeared to be negative on RT-PCR level proving that the DNAse digestion during RNA isolation was successful. β-tubulin (β-tub) was used as a control. (D) PNGase digestion of tenascin-R secreted by TNR⁺ CHO cells and the TNR⁺ ESC clone 6B showed a shift from a high molecular weight form down to 150 kDa pointing towards a strong N-glycosylation of tenascin-R in both eukaryotic cell types.
Interestingly, the size of the tenascin-R protein in undifferentiated TNR⁺ ESCs and also in transfected CHO cells differed from its typical size of 160 and 180 kDa (fig. 5 B; Pesheva et al., 1989). A reason for that could be that glycosylation of tenascin-R was enhanced in these cells. To test this, the medium supernatant of transfected CHO and of transfected ES cells was digested with PNGase F that specifically removes N-glycosidically linked sugar residues from proteins (fig. 5 D). In both groups the tenascin-R band shifted down to 150 kDa as it has been described for PNGase F-treated tenascin-R protein produced by the human neuroblastoma cell line SH-SY5Y in vitro (Woodworth et al., 2004). These data indicate that posttranslational N-glycosylation of tenascin-R was abundant in TNR⁺ CHO and undifferentiated TNR⁺ ES cells.

4.2 TNR⁺ ESCs express tenascin-R on a high level throughout in vitro-differentiation into neural precursor and postmitotic cells.

One aim of this study was to analyze if an enhanced expression of tenascin-R alters the differentiation and migration behaviour of embryonic stem cells in vitro and in vivo. Thus, a stable and high expression of tenascin-R during in vitro-differentiation was an important prerequisite for experiments addressing this question. ES cells were subjected to the five-stage differentiation protocol (Lee et al., 2000) with minor modifications.

Cells and medium supernatant were collected throughout differentiation at stages 1, 4 and 5 and RT-PCR and Western blot analysis were performed as described above (fig. 6). In contrast to TNR⁻ ESCs cells transgenic TNR⁺ stem cells expressed tenascin-R protein at high levels at the undifferentiated stage (stage 1), at the stage of neural precursor cells (stage 4) and also at the stage of postmitotic neuronal and glial cells types (stage 5). Interestingly, tenascin-R was expressed as different glyco- and isoforms throughout differentiation (fig. 6 A). At stage 1 tenascin-R presented as a single band with a high molecular weight in Western blot analysis as described above. In contrast, the tenascin-R protein expressed by TNR⁺ neural precursor cells and by TNR⁺ postmitotic neural cells (stages 4 and 5) appeared as the two typical isoforms with a molecular weight of 160 kDa and 180 kDa.

The constant expression of tenascin-R throughout differentiation was verified on RNA-level by RT-PCR. As expected, the transgenic construct was expressed on RNA-level in TNR⁺ cells at stages 1, 4 and 5 in contrast to the negative control (fig. 6 B). Thus, a stably transfected embryonic stem cell line was generated in order to unravel the effects of tenascin-R on
differentiation and migration of cells *in vitro* and *in vivo* after transplantation into an animal model of Huntington’s disease.

**Figure 6:** Transfected embryonic stem cells express tenascin-R at all stages of differentiation. Embryonic stem cells were transfected with an expression vector encoding tenascin-R (TNR+) or the empty vector alone (TNR-) and differentiated according to a protocol that covers 5 stages. (A) Western blot analysis detecting tenascin-R protein was performed from medium supernatant from TNR+ and TNR- clones at stages 1, 4, and 5. The TNR+ clone expressed high levels of tenascin-R protein in the typical isoforms of 160 and 180 kDa at stages 4 and 5 in contrast to the negative control. (B) RT-PCR analysis verified the expression of the transgenic tenascin-R in TNR+ but not in TNR- cells at stages 1, 4 and 5. RT-PCR analysis of β-tubulin is shown as a control.

**4.3 Differentiation of TNR+ and TNR- ESCs into neural cell types *in vitro.*

**4.3.1 TNR+ ESCs showed unaltered differentiation into neural precursor cells *in vitro* (stage 4)**

First, early neural differentiation was analyzed in TNR+ and TNR- cultures. For that, differentiating embryonic stem cells were stained at stage 4 with an antibody directed against the cytoskeletal and neural precursor cell marker protein nestin (fig. 7 A). Both, TNR+ and TNR- ESCs differentiated into nestin+ cells with a bipolar shape. Quantification revealed that the tenascin-R protein did not influence the differentiation of ESCs into nestin+ neural precursor cells with 61,0 ± 4,6% of all TNR+ cells and 65,9 ± 5,7% of all TNR- cells being nestin+ (fig. 7 B).
Figure 7: Tenascin-R does not influence differentiation of ESCs into neural precursor cells in vitro. (A) Immunostainings for the neural precursor cell marker nestin (red) were performed on eGFP+ (green) TNR+ and TNR- cells at stage 4 of in vitro-differentiation. Co-labeled cells appear yellow. Scale bar, 100µm. eGFP, enhanced GFP. (B) Percentages of nestin+ cells of all eGFP+ cells are shown. TNR+ and TNR- differentiated to the same extent into nestin+ neural precursor cells. The graph shows mean values ± standard error of the mean (s.e.m.).

Because enhanced or decreased proliferation and apoptosis of cells could affect their further differentiation into postmitotic neural cells, differentiating cells were labeled at stage 4 with 10µM BrdU for 8 hours and were immunostained afterwards for BrdU to assess proliferation (fig. 8 A and B). In addition, these cells were also stained for caspase-3 in order to analyze apoptosis of cells (fig. 9 A and B). About 59,8 ± 3,5% of TNR+ cells and 58,7 ± 7,3% of TNR- cells were positive for BrdU (fig. 8 A and B) whereas a minority of TNR+ and TNR- cells were positive for caspase 3 (4,3 ± 1,2% versus 3,8 ± 0,5% caspase-3+ cells of all eGFP+ cells; fig. 9 A and B). These data indicate that an enhanced production of tenascin-R did not influence proliferation and apoptosis of differentiating ESCs in vitro.
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4.3.2 Tenascin-R enhances neuronal differentiation of ESCs in vitro (stage5)

After withdrawal of FGF-2 neural precursor cells started to differentiate into βIII-tubulin⁺ postmitotic neurons, GFAP⁺ (glial fibrillary acidic protein) astrocytes and CNPase⁺ (cyclic nucleotide phosphodiesterase) oligodendrocytes (fig. 10). Quantification of differentiation at day 7 of stage 5 revealed that 32,5 ± 3,2% of all TNR⁺ cells but only 25,7 ± 2,5% of all TNR⁻ cells differentiated into βIII-tubulin⁺ neurons indicating that tenascin-R induced neuronal differentiation of ESCs in vitro. In contrast, the differentiation into GFAP⁺ astrocytes (49,9 ±
2.3% of all TNR<sup>+</sup> cells versus 57.1 ± 2.3% of all TNR<sup>-</sup> cells) and CNPase<sup>+</sup> oligodendrocytes (12.1 ± 0.9% of all TNR<sup>+</sup> cells versus 8.6 ± 2.6% of all TNR<sup>-</sup> cells) was not significantly altered in the presence of secreted tenascin-R although a tendency towards decreased astrocytic differentiation could be seen in TNR<sup>+</sup> cells versus TNR<sup>-</sup> cells. A minority of cells still remained nestin<sup>+</sup> at this stage (5.5 ± 1.1% of all TNR<sup>+</sup> versus 8.7 ± 3.8% of all TNR<sup>-</sup> cells).

Figure 10: Tenascin-R enhances neuronal differentiation of ESCs in vitro. Generation of neurons (A), astrocytes (C) and oligodendrocytes (E) from TNR<sup>+</sup> and TNR<sup>-</sup> ESCs was determined at day 7 of stage 5 by immunostaining for βIII-tubulin (A), GFAP (C) or CNPase (E), respectively (red). eGFP<sup>+</sup> cells, green. Co-labeled cells appear yellow in merged images. (B) The proportion of βIII-tubulin<sup>+</sup> cells of all eGFP<sup>+</sup> cells was greater in TNR<sup>+</sup> cultures than in TNR<sup>-</sup> cultures whereas the proportion of GFAP<sup>+</sup> (D) and CNPase<sup>+</sup> (F) cells of all eGFP<sup>+</sup> cells differed only slightly between TNR<sup>+</sup> and TNR<sup>-</sup> cultures. Scale bar, 100µm. All graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (**p: 0.01).
Additionally, the differentiation into GABAergic, dopaminergic and cholinergic neuronal subtypes was analyzed using antibodies directed against glutamic acid decarboxylase (GAD), tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT), respectively (fig. 11). The percentage of positive cells was calculated in relation to βIII-tubulin+ neurons. Both, TNR+ and TNR− cells preferentially differentiated into GAD+ GABAergic neurons (TNR+: 78.9 ± 2.5% of all βIII-tubulin+ neurons; TNR−: 79.2 ± 2.4% of all βIII-tubulin+ neurons) whereas a smaller portion of neurons became TH+ dopaminergic (TNR+: 5.8 ± 1.8% of all βIII-tubulin+ neurons; TNR−: 6.9 ± 4.4% of all βIII-tubulin+ neurons) or cholinergic (TNR+: 2.1 ± 1.4% of all βIII-tubulin+ neurons; TNR−: 2.3 ± 0.9% of all βIII-tubulin+ neurons).

These data show that tenascin-R significantly enhanced neuronal differentiation of embryonic stem cells at stage 5 but no difference regarding relative neuronal subtype specification could be observed between TNR+ and TNR− cells with a predominant GABAergic differentiation in both groups.

**Figure 11:** Tenascin-R does not influence neuronal subtype specification. Immunostainings of eGFP+ TNR+ and TNR− cells (green) at day 14 of stage 5 for (A) glutamic acid decarboxylase (GAD) and (B) tyrosine hydroxylase (TH) show GABAergic and dopaminergic differentiation (red). Co-labeled cells appear yellow in merged images. (C) Percentages of GAD+, TH+ and choline acetyltransferase (ChAT)-positive neurons of all βIII-tubulin+ neurons as determined by immunostaining are displayed. No differences in the subtype differentiation into GABAergic, dopaminergic and cholinergic neurons could be observed. The graph shows mean values ± s.e.m.
4.4 Migration of differentiated TNR$^+$ and TNR$^-$ ESCs \textit{in vitro}

As a matrix molecule tenascin-R might influence the migration of differentiating embryonic stem cells \textit{in vitro}. In order to test this, a scratch assay was performed with TNR$^+$ and TNR$^-$ cells differentiated to stage 4 (fig. 12 A). After TNR$^+$ or TNR$^-$ cells had been grown to confluency, a cell-free space was produced by performing a single scratch with a sterile pipette tip. After 48 hours in the absence of FGF-2 the number of cells was measured per visual field in the scratched and non-scratched areas (fig. 12 B, C and D). Whereas 62,8 ± 9,5 TNR$^-$ cells (per visual field) migrated into the scratched area only 13,6 ± 4,9 TNR$^+$ cells (per visual field) performed such migration showing that tenascin-R inhibited the migration of neural precursor cells \textit{in vitro}. In order to verify that the inhibition of migration was tenascin-R-dependent, mixed cultures were performed in this scratch assay ranging from 25% TNR$^+$ cells (and 75% TNR$^-$ cells) to 50% TNR$^+$ cells (and 50% TNR$^-$ cells) and 75% TNR$^+$ (and 25% TNR$^-$ cells). Analysis showed that the number of cells in the scratched area was higher when the number of TNR$^+$ cells was lower (fig. 12 C). To rule out that these differences in migration were due to a decreased density of cells in stage 4 cultures, the density of TNR$^+$ and TNR$^-$ cells were also analysed in non-scratched areas. Importantly, no differences in cell density could be observed between TNR$^+$ and TNR$^-$ cultures in non-scratched areas (305 ± 33 TNR$^+$ cells versus 328 ± 36 TNR$^-$ cells per visual field).

These data indicate that tenascin-R inhibits the migration of differentiating stem cells in a concentration-dependent manner \textit{in vitro}.
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Figure 12: TNR⁺ cells show decreased migration in vitro.

(A) To analyze the effect of tenasin-R on migration of transfected stem cells in vitro, a scratch assay was performed. TNR⁺ or TNR⁻ cells differentiated to stage 4 were grown to confluency. A cell-free space was produced by performing a single scratch. After 48 hours in the absence of FGF-2, the number of cells was measured per visual field in the scratched and non-scratched areas. Experiments were performed with mixed cultures ranging from 100% TNR⁺ cells to 100% TNR⁻ cells. (B) Confocal images of scratched cultures consisting of 100% TNR⁺ or 100% TNR⁻ cells (green) 48 h after scratching. The white lines mark the border between scratched and non-scratched area. In TNR⁺ cultures less eGFP⁺ cells could be observed in the scratched area as compared to the TNR⁻ cultures. Scale bar, 100 µm. (C) Quantification of eGFP⁺ cells in the scratched area in cultures consisting of 100% TNR⁺ cells, of 100% TNR⁻ cells or of mixed cultures revealed a tenascin-R-dependent inhibition of migration. Significant differences could be observed between cultures consisting of 100% TNR⁺ and 100% TNR⁻ cells. Student’s t test was performed for statistical analysis (**p: 0.01). (D) The number of eGFP⁺ cells in non-scratched areas did not differ between 100% TNR⁺ and 100% TNR⁻ cultures excluding that the observed difference in migration was due to different cell densities in the non-scratched areas. All graphs show mean values ± s.e.m.
4.5. Histological presentation and in vivo-imaging of the mouse striatum lesioned with quinolinic acid

The induction of excitotoxicity is a well-established method to generate a suitable model of Huntington’s disease in rodents (Isacson et al., 1984; Beal et al., 1991). Quinolinic acid (QA) is a NMDA receptor agonist that leads to excitotoxicity and cell death of GABAergic medium-sized spiny projection neurons when directly injected into the striatum of healthy mice. Three days after unilateral intrastriatal injection of QA a reactive astrogliosis and an activation of resting microglia could be observed in the lesioned but not in the contralateral striatum (fig. 13 A and B). Since the GABAergic medium-sized projection neurons make up about 90% of all striatal neurons, a massive loss of NeuN+ neurons occurred in the lesioned striatum whereas no changes in NeuN+ neurons were observed on the contralateral side (fig. 13 C). Further characterization of the lesion revealed that the damaged neurons were positive for glutamic acid decarboxylase (GAD) and thus constituted damaged GABAergic inhibitory neurons (fig. 13 D). Magnetic resonance images (MRI) of the brain of QA-lesioned mice revealed that in accordance to these excitotoxic events a hyperintense edema developed within 3 days after injection of QA that appeared to be restricted to the striatum (fig. 13 E). The size of this edema declined within the first week after injection of QA and hyperintensity of striatal tissue became restricted to an area around the injection canal. This canal gave a hypointense signal possibly due to the presence of remaining air within its lumen (fig. 13 F). Interestingly, ongoing excitotoxicity-induced tissue damage could be visualized with MRI scans over a period up to 28 days after QA-lesion (fig. 14). In contrast to 7 days after lesion, a hypointensity and an obvious shrinkage of the lesioned striatum could be seen 14 days and even more pronounced 28 days after injection of QA. This tissue damage was accompanied by an enlargement of the ipsilateral lateral ventricle.

These morphological data show that the QA mouse model of Huntington’s disease is suitable for analyzing a cell replacement strategy that aims to repopulate the damaged striatum with inhibitory GABAergic neurons introduced by stereotaxic intrastratal injection.
Figure 13: Intrastriatal injection of quinolinic acid leads to degeneration of GABAergic neurons.

(A) Quinolinic acid (QA) was stereotaxically injected into the right striatum of adult wildtype mice. 
(B) Three days after injection immunostainings of the lesioned and of the unlesioned striatum were performed with antibodies directed against GFAP marking reactive astrocytes (red) or Iba-1 detecting activated microglia (red). Note the activation of both cell types within the lesion. Scale bar, 100µm (C) Immunostaining for NeuN visualizes the loss of postmitotic neurons within the lesioned striatum. (D) Degenerated neurons had a GABAergic specification as proven by immunostainings of the lesion site for GAD. Scale bar, 100µm. (E and F) Horizontal magnetic resonance images of a mouse head 3 (E) and 7 (F) days after injection of QA. A hyperintense (white) edema restricted to the ipsilateral striatum was visible 3 days after injection that could not be observed 7 days after treatment. The hypointense (black) area marks the injection canal that is surrounded by a hyperintense ring of degenerating tissue.
Figure 14: MRI scans revealed progressive striatal tissue damage after injection of QA. Horizontal MRI scans through a mouse head were performed 7 (A), 14 (B) and 28 (C) days after injection of QA into the right striatum. In contrast to the contralateral side, the lesioned striatum showed a shrinkage of hypointense (dark grey) tissue 14 and more pronounced 28 days after lesion. As a consequence the ipsilateral lateral ventricle was enlarged.

4.6 Stable expression of tenascin-R after transplantation of TNR$^+$ cells into QA-lesioned mice

It was very important that TNR$^+$ cells expressed high amounts of tenascin-R also after transplantation into QA-lesioned animals. Since adult wildtype mice express tenascin-R in the forebrain including the striatum, 200,000 pre-differentiated TNR$^+$ or TNR$^-$ cells were unilaterally engrafted into TNR-deficient mice 3 days after a lesion with QA to verify stable expression of tenascin-R also in vivo (fig. 15 A). One month after engraftment mice were sacrificed and intrastriatal eGFP$^+$ grafts were isolated from extracted brains under a fluorescence microscope for Western blot analysis. Importantly, tenascin-R was abundantly expressed by implanted TNR$^+$ but not by implanted TNR$^-$ cells (fig. 15 B).

Next, pre-differentiated TNR$^+$ and TNR$^-$ cells were engrafted into wildtype animals 3 days after QA-lesion and expression of tenascin-R was analyzed on a histological level 2 months after transplantation (fig. 15 C). Again, engrafted TNR$^+$ cells expressed tenascin-R on a high level whereas TNR$^-$ cells showed only poor expression of tenascin-R.

These data demonstrate that the experimental design was suitable to unravel effects of tenascin-R on stem cell-mediated neuroregeneration in QA-lesioned animals as will be presented in the following chapters.
Figure 15: Tenascin-R is stably expressed by TNR+ cells up to 2 months after transplantation into quinolinic acid-lesioned animals. (A) Embryonic stem cells differentiated to day three of stage 5 were transplanted into the mouse striatum three days after quinolinic acid (QA)-induced lesion. (B) For analysis of tenascin-R expression in the grafts, TNR+ and TNR- cells were transplanted into mice deficient for tenascin-R. Western blot analysis of tenascin-R expression in the grafts one month after transplantation is displayed. Note the stable expression of tenascin-R in the TNR+ graft. Western blot analysis of β-tubulin is shown as a loading control. (C) Immunostaining of TNR+ and TNR- grafts (green) for tenascin-R two months after transplantation (red) into QA-lesioned wildtype mice. Scale bar, 100 µm.

4.7 Transplantation of pre-differentiated ESCs into QA-lesioned mice

After induction of terminal differentiation for 3 days in stage 5 in vitro, 100,000 pre-differentiated TNR+ and TNR- cells were stereotaxically engrafted into the right striatum of wildtype C57BL/6J mice that have been lesioned with QA 3 days prior to transplantation as described above. For histological examination mouse brains were analyzed 1 and 2 months after transplantation. Stereological analysis of engrafted cells was performed using a Neurolucida-software-controlled microscopic device. Immunohistological analysis was performed applying confocal laser scanning microscopy.
4.7.1 Grafts consisting of TNR\(^+\) or TNR\(^-\) cells did not differ in size and number of engrafted cells

As seen in figure 15 engrafted eGFP\(^+\) cells were clearly distinguishable from eGFP\(^-\) host cells. The size of grafts and the number of eGFP\(^+\) cells within the grafts did not differ between the TNR\(^+\) and the TNR\(^-\) group 1 month and 2 months after transplantation (fig. 16 A and B). As a consequence also the density of cells within the grafts did not differ significantly in the TNR\(^+\) and the TNR\(^-\) group (fig. 16 C). Importantly, grafts remained viable up to 2 months after transplantation as indicated by the stability of parameters described here.

Figure 16: Grafts consisting of TNR\(^+\) or TNR\(^-\) cells did not differ in size and number of engrafted cells.
Stereological analysis was performed on TNR\(^+\) and TNR\(^-\) grafts 1 and 2 months after transplantation of pre-differentiated stem cells into QA-lesioned animals. The total number of engrafted eGFP\(^+\) cells (A), the volume of grafts (B) and the density of engrafted cells (C) did not differ between the TNR\(^+\) and the TNR\(^-\) group 1 and 2 months after transplantation. All graphs show mean values ± s.e.m.
4.7.2 Neuronal differentiation was enhanced in grafts consisting of TNR\(^+\) cells

In order to analyze the effect of tenascin-R on neuronal, astrocytic and oligodendrocytic differentiation of engrafted eGFP\(^+\) cells \textit{in vivo}, coronal sections were stained with antibodies directed against the neuronal marker protein NeuN (neuronal nuclear antigen), against GFAP and against CNPase (fig. 17). The percentage of NeuN\(^+\) of all engrafted cells was significantly higher in grafts consisting of TNR\(^+\) cells when compared to grafts that consisted of TNR\(^-\) cells (fig. 17 A and B). One month after transplantation 39,9 ± 3,9% of all TNR\(^+\) cells were NeuN\(^+\) neurons whereas only 22,9 ± 2,5% of all TNR\(^-\) cells had differentiated into NeuN\(^+\) cells. Similarly, a tenascin-R-mediated two-fold increase in neuronal differentiation was observed 2 months after transplantation. At this time point 30,8 ± 3,5% of all engrafted TNR\(^+\) but only 14,7 ± 1,9% of all engrafted TNR\(^-\) cells were stained for NeuN.

In contrast, astrocytic differentiation was decreased in grafts consisting of TNR\(^+\) cells when compared to grafts consisting of TNR\(^-\) cells (fig. 17 C and D). One month after transplantation only 34,7 ± 3,1% of all TNR\(^+\) cells were GFAP\(^+\) astrocytes whereas 54,9 ± 5,8% of all TNR\(^-\) cells had differentiated into GFAP\(^+\) cells. Two months after transplantation 34,1 ± 6,5% of all TNR\(^+\) cells but 47,1 ± 5,3% of all TNR\(^-\) cells were positive for GFAP. Statistical analysis revealed that differences in astrocytic differentiation were significant only 1 month after transplantation.

Oligodendrocytic differentiation was poor in both groups at both time points with levels of differentiation below 4% (fig. 17 E and F). Significant differences could not be observed between the TNR\(^+\) and the TNR\(^-\) group.
Figure 17: TNR+ cells show increased neuronal and decreased astrocytic differentiation after transplantation into the quinolinic acid-lesioned mouse striatum (A). Laser scanning microscopy illustrating immunohistochemical analysis of grafts of TNR+ and TNR- cells with the neuronal marker NeuN (red) 1 month after transplantation of eGFP+ (green) cells. Donor-derived marker+ cells in merged images appear yellow. Note the increased expression of NeuN in TNR+ grafts when compared with TNR- grafts. (B) Percentages of NeuN+ cells of grafted eGFP+ cells one month and two months after transplantation. (C) Immunohistochemical analysis of the grafts (green) with the astroglial marker GFAP (red) one month after transplantation. Note the decreased number of GFAP+ cells in the TNR+ grafts when compared to TNR- grafts. (D) Percentages of GFAP+ cells of grafted eGFP+ cells one month and two months after transplantation. (E) Immunohistochemical analysis of the grafts with antibodies directed against the oligodendrocytic marker protein CNPase (red) one month after transplantation. Scale bar 100 µm. (F) Percentages of CNPase+ cells of grafted eGFP+ cells one month and two months after transplantation. All graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.05; **p: 0.01).
Next, the absolute number of NeuN+ neurons was counted in TNR+ and TNR- grafts 1 and 2 months after transplantation (fig. 18). One month after transplantation grafts consisting of TNR+ cells contained 38870 ± 7098 NeuN+ neurons whereas TNR- grafts contained significantly less (15450 ± 3371) NeuN+ neurons. Similarly, 38450 ± 19320 NeuN+ neurons were found in TNR+ grafts but only 16960 ± 4652 NeuN+ neurons in TNR- grafts 2 months after transplantation. Due to a higher variability in the number of neurons in TNR+ grafts 2 months after injection, differences in the total number of neurons were significant only 1 month after transplantation.

**Figure 18:** TNR+ grafts contained more NeuN+ positive neurons than TNR- grafts. The total number of NeuN+ neurons was counted in TNR+ and TNR- grafts 1 and 2 months after transplantation of pre-differentiated stem cells into QA-lesioned mice. The presence of tenascin-R led to enhanced numbers of eGFP+ and NeuN+ postmitotic neurons in the grafts. The graph shows mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.05).

Since in the QA mouse model of Huntington’s disease the GABAergic medium-sized spiny projection neurons are depleted (chapter 4.5) it was interesting to know if the graft-derived neurons acquired a GABAergic phenotype. To test this, sections were stained with an antibody detecting GAD as a marker protein for GABAergic differentiation (fig.19 A and B). One and two months after transplantation the total number graft-derived GABAergic cells and also the percentage of graft-derived GAD+ neurons of all engrafted cells were determined (fig. 19 C and D). One month after transplantation 20420 ± 5169 GAD+ neurons (17,6% of all GFP+ cells) were observed in TNR+ grafts whereas TNR- grafts contained significantly less GAD+ neurons (7116 ± 829 cells) constituting 10,7% of all GFP+ cells (fig. 19 C and D). Two
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months after transplantation 22740 ± 10290 GAD⁺ neurons were counted in TNR⁺ grafts (15.1% of all GFP⁺ cells) versus 13950 ± 5893 GAD⁺ neurons (10.8% of all GFP⁺ cells) in grafts consisting of TNR⁻ cells.

Finally, the percentage of GAD⁺ GABAergic neurons of all graft-derived NeuN⁺ neurons was measured in TNR⁺ and TNR⁻ grafts (fig. 19 E). In both groups about the half of graft-derived neurons were GABAergic 1 month after engraftment (52.8 ± 7.8% of TNR⁺ neurons versus 52.4 ± 8.2% of TNR⁻ neurons) and their percentage raised 2 months after transplantation to 62.8 ± 12.9% (TNR⁺ neurons) and 73.5 ± 15.3% (TNR⁻ neurons), respectively.

These results show that the secreted tenasin-R protein caused an almost twofold increase in neuronal differentiation of ESC-derived neural precursor cells after transplantation in a mouse model of Huntington’s disease. Importantly, a high percentage of these neurons was GABAergic and thus, a twofold increase in the total number of GABAergic neurons was seen in TNR⁺ grafts. In contrast, astrocytic differentiation was decreased when tenascin-R was abundantly expressed by engrafted cells.
Figure 19: The number of GABAergic neurons is increased in TNR+ grafts.
(A) Laser scanning microscopy illustrating immunohistochemical analysis of grafts of TNR+ and TNR- cells with GAD (red) eight weeks after transplantation of eGFP+ (green) cells. Donor-derived GABAergic cells in merged images appear yellow. Note the increased expression of GAD in TNR+ grafts in comparison to TNR- grafts. Scale bar, 100µm. (B) Projections of stacked images of 1µm thickness of a TNR+ and a TNR- graft 2 months after transplantation. Scale bar, 50µm. (C) Percentages of GAD+ cells of engrafted eGFP+ cells one month and two months after transplantation. The percentage of GABAergic neurons in grafts was significantly enhanced in the presence of tenascin-R. (D) Quantification of the absolute number of GAD+ GABAergic neurons in grafts 1 and 2 months after transplantation. Note that the number of GAD+ GABAergic neurons was significantly enhanced in TNR+ grafts as compared to TNR- grafts. (E) Percentages of GAD+ cells of graft-derived NeuN+ neurons showing that tenascin-R did not alter relative neuronal subtype specification 1 and 2 months after transplantation. All graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.05).

4.7.3 Tenascin-R decreases the migration of engrafted cells into the striatum of QA-lesioned mice

The migration of engrafted cells into the host tissue is an important parameter for the integration of cells in the recipient brain. In order to analyze the general ability of engrafted pre-differentiated stem cells to migrate in the QA-lesioned adult forebrain, differentiating TNR- cells were labeled at stage 4 with the paramagnetic contrast dye Resovist® (Schering)
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for 24 hours. These cells were implanted 2 days after labelling into the striatum of QA-lesioned mice (fig. 20). MRI scans of the brains were performed 28 days after implantation to assess migration of labeled cells within or even outside the striatum. Engrafted cells did not perform migration over long distances in the striatum but remained at the site of implantation.

Figure 20: ESC-derived cells implanted into the QA-lesioned striatum show poor migration. Differentiating TNR− cells were labeled with a paramagnetic contrast dye containing iron particles and were afterwards engrafted into the striatum of QA-lesioned mice. Brightfield images of unstained (A) and of iron-stained (B; Turnbull) TNR− cells showed successful labeling of cells before implantation. Scale bar, 10µm. (C) Horizontal MRI scan through a mouse head 28 days after implantation of labeled cells. Note the poor migration of cells into the host striatum.

In order to unravel the effect of tenascin-R on migration of implanted pre-differentiated stem cells in QA-lesioned animals, the distance was measured at the medial, lateral and ventral aspect of the graft that eGFP+ TNR+ and TNR− cells migrated from the graft edge into the host striatum 1 and 2 months after transplantation (fig. 21). At least 100 measurements were performed for each animal. Migrating cells had an elongated shape in both groups and were easily distinguishable from striatal host cells. One month after transplantation engrafted TNR+ cells had migrated significantly shorter distances into the host striatum (60 ± 3µm) when compared to transplanted TNR− cells (82 ± 7µm). A decreased migration was also seen 2 months after transplantation (51 ± 3 µm migration of TNR+ cells versus 71 ± 4 µm migration of TNR− cells).
Figure 21: TNR+ cells show decreased migration into the host tissue after transplantation. (A) The mean distance of TNR+ and TNR- cells from the graft edge 1 month and 2 months after transplantation is shown. At both time points TNR+ cells show decreased migration as compared to TNR- cells. Student’s t test was performed for statistical analysis (*p: 0.05; **p: 0.01). (B) Confocal images of the periphery of TNR+ or TNR- grafts. White lines indicate graft edges. Scale bar, 100µm.

4.7.4 Influence of tenasin-R on synaptic coverage of engrafted and host-derived cells

A second important parameter for the integration of implanted cells in the recipient brain constitutes the synaptic connectivity between striatal host neurons and graft-derived cells. In this context, two different aspects should be regarded. On the one hand, endogenous host-derived neurons can give synaptic input to engrafted cells and on the other hand, engrafted cells can give synaptic output to host-derived neurons. Since tenasin-R is known to regulate synaptogenesis in vivo (Nikonenko et al., 2003) effects of tenasin-R on these two different aspects of synaptogenesis were analyzed 1 month after transplantation of TNR+ and TNR- cells (fig. 22).

Synaptic coverage was analyzed with confocal microscopy at the interface between the host striatum and the eGFP+ grafts after immunostaining of sections for the synaptic vesicle protein SV-2 that marks pre-synaptic boutons (fig. 22 A and B). Quantification of eGFP+ cells covered by host-derived SV-2+ synaptic boutons revealed that 6.7 ± 1.3% of all TNR+ cells and 4.4 ± 0.8% of all TNR- cells received synaptic input from host neurons (fig. 22 C). Quantification of eGFP+ and SV-2+ boutons on host-derived cells showed that in TNR+ grafts 2.7 ± 1.3% of the grafted cells and in TNR- grafts 1.5 ± 0.4% of the grafted cells had eGFP+ and SV-2+ boutons (fig. 22 D).
These results show a tendency for tenascin-R to enhance afferent and efferent synaptic connection between donor-derived and host-derived neurons in the QA model of Huntington’s disease but differences were statistically not significant.

**Figure 22:** Synaptic boutons between graft-derived and host-derived neurons. (A) Confocal image of a histological section immunostained for the synaptic vesicle protein SV-2 (red) at the interface between host striatum and engrafted TNR⁻ cells (green). Scale bar, 50 µm. (B) Higher magnification of an immunostained area for SV-2 (red) shown in (A). Note host-derived GFP⁺ and SV-2⁺ boutons (red) on the soma and neurite of an engrafted GFP⁺ cell (arrows) and a GFP⁺ and SV-2⁺ bouton (asterix) that appears yellow in the merged image. Scale bar, 20 µm. (C) Percentage of GFP⁺ cells of all engrafted cells at the graft edge covered by host-derived SV-2⁺ boutons as indication of synaptic input to engrafted cells. (D) Percentage of GFP⁺ cells with SV-2⁺ boutons of all engrafted GFP⁺ cells at the graft edge as indication of synaptic output to host-derived neurons. The difference between TNR⁺ and TNR⁻ cells is statistically not significant. Student’s *t* test was performed for statistical analysis. Shown are mean values ± s.e.m.
4.8 Host-derived neuroblasts react on the transplantation of pre-differentiated ESCs into QA-lesioned mice

The injection of quinolinic acid and the transplantation of ESC-derived neural precursor cells might have an influence on residing and migrating endogenous neural precursor cells. In order to test this, neurogenesis and migration of host-derived neuroblasts were analyzed applying immunohistochemistry with an antibody directed against the structural protein doublecortin (dcx) since it is known to be specifically expressed by migrating neuroblasts. A Neurolucida software-controlled stereological device was used for precise histological analysis.

4.8.1 The distribution of doublecortin$^+$ neuroblasts in unlesioned mice

First, the distribution of dcx$^+$ neuroblasts in the adult brain at the level of injection of QA and stem cells should be shortly presented. In untreated animals dcx$^+$ cells were found in the subventricular zone (SVZ) of the lateral ventricles as small and round cells that maintained close cellular contacts (fig. 23 A). These dcx$^+$ cells formed multiple layers within the SVZ and appeared in a higher number in the most dorsal and the most ventral part of the lateral ventricles (fig. 22A and B). In addition, dcx$^+$ cells were observed in higher numbers in the anterior parts of the SVZ. Other cell types that were negative for dcx were also present in the subventricular zone with a more prominent distribution in the posterior parts of the lateral ventricles. On the septal (medial) side of the lateral ventricles almost no dcx$^+$ cells were observed.

Dcx$^+$ cells showed migration from the SVZ at different sites. Only few cells performed direct migration into the adjacent striatum (fig. 23 A). On the ventral side of the SVZ, some dcx$^+$ cells detached in order to migrate into the septal and striatal area (fig. 23 B). However, the most prominent migration of dcx$^+$ neuroblasts could be observed in the dorsal part of the lateral ventricle (fig. 23 C). Here, round shaped dcx$^+$ neuroblasts detached from the SVZ to generate the posterior extension of the rostral migratory stream. Some of these cells migrated laterally in a niche between the dorsal part of the striatum and the inner ventral part of the corpus callosum (fig. 23 D). The number of dcx$^+$ cells declined with increasing distance from the SVZ and the cells acquired an elongated shape with a leading process pointing laterally. Interestingly, these migrating neuroblasts did not enter the adjacent striatum (fig. 23 D).
Figure 23: Distribution of doublecortin+ cells in the unlesioned adult mouse brain. (A) Immunostaining of the dorsal SVZ of an unlesioned adult mouse brain for the nuclei marker DAPI (blue) and for doublecortin (dcx), a marker for migrating neuroblasts (red). Co-labeled cells in the merged image appear purple. Scale bar, 100µm. (B) Immunostaining of the ventral SVZ of an unlesioned adult brain for DAPI (blue) and for dcx (red). Co-labeled cells in the merged image appear purple. Scale bar, 100µm. (C) Combined immunostained sections for dcx (red) showing migration of cells in the posterior extension of the rostral migratory stream with some cells performing lateral migration. Scale bar, 100µm. (D) Immunostained section for dcx (red) demonstrating a typical morphology of migrating cells. Note that these cells do not enter the adjacent striatum. Scale bar, 20µm.
4.8.2 Changes in the SVZ after intrastriatal injection of QA and transplantation of pre-differentiated ESCs

Three groups of animals were compared. Besides QA-lesioned animals that received a unilateral intrastriatal injection of TNR$^+$ or TNR$^-$ stem cells, a third group of animals was analyzed that was intrastriatally injected with PBS instead of pre-differentiated stem cells three days after QA treatment. Accordingly, this group was termed PBS. The following parameters were obtained from sections at the injection level and thus, corresponding areas were compared between the three groups.

First, the number of doublecortin$^+$ cells within the SVZ was counted on the side of treatment (ipsilateral) and on the contralateral side 1 and 2 months after operation (fig. 24 A and B). In all groups the number of dcx$^+$ cells was enhanced on the ipsilateral side 1 and 2 months after transplantation when compared to the contralateral side. However, this increase was statistically not significant.

In contrast, the mean width and mean area of the ipsilateral SVZ was significantly enhanced in the TNR$^+$, TNR$^-$ and PBS group 1 and 2 months after injection when compared to the contralateral side (fig. 24 C and D). Since no major differences were observed between the 3 groups, effects on the enlargement of the SVZ were possibly due to the QA lesion rather than due to the implantation of pre-differentiated stem cells.
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Figure 24: Intrastriatal lesion with QA leads to an enlargement of the ipsilateral SVZ.

(A) The number of dcx+ cells within the SVZ and its size were measured on the ipsi- and contralateral side 1 and 2 months after implantation of TNR+ or TNR- cells or after injection of PBS into the striatum of QA-lesioned animals. (B) Quantification of the numbers of dcx+ cells on the ipsi- (left column) and contralateral (right column) SVZ showed a tendency towards increased cell numbers on the ipsilateral side in the TNR+, in the TNR- and in the PBS group at both time points. Measurement of the width (C) and the area (D) of the ipsi- and contralateral SVZ showed a significant increase of both parameters on the ipsilateral side in all 3 groups 1 and 2 months after treatment. All graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.05; **p: 0.01; ***p: 0.001).
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4.8.3 Migration of endogenous neuroblasts after intrastriatal injection of QA and transplantation of pre-differentiated ESCs

As the intrastriatal implantation of pre-differentiated stem cells might influence migration of host-derived neuroblasts, migration of these dcx+ cells was analyzed at three different sites within and close to the adult striatum. The migration of host-derived neuroblasts was measured along the SVZ, within the posterior extension of the rostral migratory stream below the corpus callosum and as a detachment from the migratory stream towards the engrafted striatum.

4.8.3.1 Effects of engrafted stem cells on migration of host-derived neuroblasts along the SVZ

In the unlesioned adult brain dcx+ cells perform direct migration from the SVZ into the adjacent striatum (see chapter 4.8.1). Thus, effects of the QA-lesion and the implantation of pre-differentiated stem cells on direct intrastriatal migration were analyzed on the ipsi- and on the contralateral side 1 and 2 months after treatment (fig. 25 A and B). Interestingly, in the TNR+, TNR- and also in the PBS group the number of migrating host-derived dcx+ neuroblasts was significantly increased 1 month after treatment on the ipsilateral side when compared to the contralateral hemisphere (fig. 25 C). This effect was also observed 2 months after treatment in all three groups. Importantly, there was no difference between the stem cell groups and the PBS group and thus, effects on direct migration were mediated by the injection of QA rather than by the treatment with stem cells.

Measuring the distance between the SVZ and the graft or lesion, respectively, revealed a relatively high distance of 843 ± 81µm for TNR+ grafts, 551 ± 105µm for TNR- grafts and 689 ± 64µm for the PBS-injected lesion area that could explain for the lack of a stem cell effect on direct migration of endogenous neuroblasts in this paradigm (fig 25 D).
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Figure 25: Direct migration of host-derived doublecortin+ neuroblasts from the SVZ into the adjacent striatum.

(A) The migration of host-derived dcx+ neuroblasts cells into the adjacent striatum was analyzed 1 and 2 months after transplantation of TNR+ or TNR- cells or after injection of PBS into the striatum of QA-lesioned animals. (B) Confocal images of histological sections immunostained for dcx (red) of the ipsilateral SVZ and the adjacent striatum 1 month after engraftment of TNR+ or TNR- cells or after injection of PBS. Scale bar, 100µm. (C) Quantification of dcx+ cells migrated into the ipsilateral and contralateral striatum in the TNR+, the TNR- and the PBS group 1 and 2 months after treatment. In all three groups migration of host-derived cells was enhanced at both time points. (D) Distances between the SVZ and the medial graft edge or the medial boundary of the lesion, respectively, in the TNR+, the TNR- and the PBS group. All graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.05; **p: 0.01; ***p: 0.001).
4.8.3.2 Effects of engrafted stem cells on migration of host-derived neuroblasts within the migratory stream

Next, the number of dcx⁺ neuroblasts in the migratory stream was counted on the ipsi- and on the contralateral side 1 and 2 months after injection of TNR⁺ cells, TNR⁻ cells or PBS (fig. 26 A and B). Interestingly, the cell number was slightly but not significantly enhanced on the ipsilateral side in both stem cell groups 1 and also 2 months after engraftment (fig. 26 B).

In order to measure lateral migration towards the injection site between the corpus callosum and the striatum, the distance that host-derived neuroblasts migrated laterally was analyzed in the ipsi- and in the contralateral hemisphere 1 and 2 months after treatment with TNR⁺ cells, TNR⁻ cells or with PBS (fig. 26 C). In contrast to the PBS control, both TNR⁺ and TNR⁻ cells significantly increased ipsilateral migration of host-derived dcx⁺ cells 2 months after implantation with a mean distance of 937µm in the TNR⁺ group (649µm contralateral) and 683µm in the TNR⁻ group (505µm contralateral). Interestingly, this effect was already observed 1 month after transplantation in the TNR⁺ group only (968µm ipsilateral versus 673µm contralateral) pointing towards an accelerating effect of tenascin-R on the attraction of endogenous neuroblasts.

In order to compare the three groups, the relative enhancement of ipsilateral migration (distance migrated ipsilaterally divided by distance migrated contralaterally) was determined for each animal (fig. 26 D). One-way analysis of variance with post-hoc Turkey test revealed a significant relative enhancement of ipsilateral migration of host-derived neuroblasts in the migratory stream for the TNR⁺ and also for the TNR⁻ group 2 months after transplantation in comparison to the PBS sham control. This enhancement was already present in the TNR⁺ group 1 month after transplantation. Importantly, no enhancing effect could be observed for the PBS group 1 and 2 months after treatment.

These results show that engrafted ESC-derived cells had an attracting effect on laterally migrating endogenous neuroblasts in the migratory stream. Furthermore, this effect appeared earlier in the TNR⁺ group.
Figure 26: Tenascin-R promotes lateral migration of host-derived doublecortin+ cells in the migratory stream below the corpus callosum. 
(A) The number of host-derived dcx+ cells in the migratory stream and their lateral migration were measured 1 and 2 months after transplantation of TNR+ or TNR- cells or after injection of PBS into the striatum of QA-lesioned animals. (B) Quantification of dcx+ cells in the ipsi- and contralateral migratory stream showed some enhancement of cell number on the ipsilateral side in the TNR+ and in the TNR- group. (C) Distance of host-derived dcx+ cell migration laterally between the corpus callosum and the striatum on the ipsilateral and contralateral hemisphere. In contrast to the PBS control, both TNR+ and TNR- cells increased lateral migration of dcx+ cells. This effect was observed in the TNR+ group already 1 month after transplantation. (D) Relative enhancement of lateral migration was measured by dividing the distance that cells migrated ipsilaterally by the distance that cells migrated contralaterally. Tenascin-R accelerated the stem cell-mediated enhancement of lateral migration of host-derived cells from the rostral migratory stream towards the engrafted striatum. All graphs show mean values ± s.e.m. Student’s t test (C) and one-way analysis of variance with post-hoc Turkey test (D) were performed for statistical analysis (*p: 0.05; **p: 0.01; ***p: 0.001).
4.8.3.3 Effects of engrafted stem cells on radial migration of host-derived neuroblasts from the migratory stream

Furthermore, it was investigated whether host-derived neuroblasts detach from the migratory stream and migrate radially towards the intrastriatal grafts consisting of TNR+ or TNR- cells. To test this, the number of dcx+ cells was analyzed in an area measuring 200 x 200 µm between the corpus callosum and the intrastriatal graft or lesion, respectively. Additionally, the number of dcx+ cells was measured in a corresponding area on the contralateral side (fig. 27 A and B). Only dcx+ but GFP- cells were counted on the ipsi- and the contralateral side 1 and 2 months after treatment (fig. 27 C). Negativity for GFP was verified using laser scanning microscopy.

As seen in unlesioned animals, almost no dcx+ cells detached from the subcallosal migratory stream on the contralateral side in the TNR+, TNR- and also in the PBS group 1 and 2 months after treatment (fig. 27 C). On the ipsilateral side however, host-derived dcx+ neuroblasts detached from the migratory stream when mice had been engrafted with TNR+ or with TNR- cells (fig. 27 A and C). These dcx+ cells entered the adjacent striatum towards the engrafted eGFP+ cells and had a typical morphology of migrating cells with an ovoid cell shape and a leading process (fig. 27 A). In both, the TNR+ and the TNR- group their number within the striatum was significantly enhanced at both time points when compared to the contralateral side. Such an attractant effect on endogenous dcx+ neuroblasts was not observed in the PBS control group 1 and also 2 months after injection. Interestingly, significant differences could be observed between the TNR+ and the PBS group 1 and also 2 months after treatment whereas no significant differences appeared between the TNR- and the PBS group at both time points. Moreover, significant differences between the TNR+ and the TNR- group could be observed 2 months after implantation. For statistical analysis, two-way analysis of variance with post-hoc Turkey test was performed.

These findings demonstrate that the engraftment of ESC-derived neural cells induced radial migration of endogenous neuroblasts from the migratory stream towards the engrafted cells. Furthermore, tenascin-R was shown to favour this recruitment up to 2 months after transplantation.
Figure 27: Tenascin-R enhances stem cell–mediated attraction of endogenous doublecortin⁺ neuroblasts from the posterior extension of the rostral migratory stream.

(A) Immunostaining against doublecortin (red) of the dorsal striatum of mice two months after grafting of TNR⁺ cells (green), TNR⁻ cells (green) or sham-injection with PBS. The number of endogenous dcx⁺ cells in a defined area (B) measuring 200µm x 200µm between corpus callosum (cc) and graft (green) or corresponding area in the sham-injected animals was observed. Note the increased number of dcx⁺ cells in animals grafted with TNR⁺ cells when compared with TNR⁻ cells or sham-injected animals. (C) The number of host-derived dcx⁺ cells in the area of 200µm x 200µm defined in (B) was determined in serial sections on the side ipsi- and contralateral to the lesion 1 and 2 months after treatment. In contrast to the PBS control TNR⁺ and TNR⁻ cells significantly attracted dcx⁺ cells from the migratory stream as compared to the contralateral side. Note that only TNR⁺ cells but not TNR⁻ cells showed a significantly enhanced recruitment of host-derived dcx⁺ cells ipsilateral to the lesion when compared with the PBS control 1 and 2 months after transplantation and that TNR⁺ cells showed a more pronounced recruitment of endogenous precursor cells compared to TNR⁻ cells 2 months after transplantation. Mean values ± s.e.m. are displayed. Two-way analysis of variance with post-hoc Turkey test was performed for statistical analysis (*p: 0.05; ***p: 0.001).
4.8.4 Host-derived newborn neurons were observed within the grafted area

As shown above, newborn neurons were generated in the SVZ that migrated towards the graft after implantation of ESC-derived neural precursor cells. In order to determine if host-derived neuroblasts were also observed within the graft, dcx-stained sections of the grafts and the lesion were analyzed 1 and 2 months after transplantation or sham injection (fig. 28). In the center and the periphery of both, TNR+ and TNR- grafts strings of dcx+ cells were detected (Fig. 28 A). 100 % of TNR+ grafts and 80 % of TNR- grafts contained these dcx+ strings 1 month after engraftment and even 2 months after transplantation such strings could still be detected in 50 % of TNR+ grafts and in 50 % of TNR- grafts (Fig. 28 B). These strings only partly overlapped with an expression of eGFP showing that host-derived newborn neurons were found within the grafted areas. Importantly, in PBS-treated lesioned mice such dcx+ strings of cells could not be observed.

Figure 28: Host-derived doublecortin+ newborn neurons were present in the grafted striatum. (A) Confocal images of histological sections of TNR+ and TNR- grafts (green) and the PBS-injected lesioned striatum immunostained for dcx (red) 1 month after treatment. In merged images graft-derived dcx- newborn neurons appear yellow whereas host-derived dcx- newborn neurons appear red. Scale bar, 100µm. (B) Percentages of grafts containing dcx- strings of cells of all grafts 1 and 2 months after transplantation. Note that dcx- strings of cells are absent in the PBS group. Data from one experiment are shown. (C) Schematic presentation of analyzed area.
As a second test for the recruitment of host-derived newborn neurons in the grafted area, QA-lesioned mice were labeled with bromodesoxyuridine (BrdU) from day 3 to day 7 after transplantation of cells or after injection of PBS, respectively. Brain sections including the injection site were stained for BrdU and NeuN 1 month after transplantation (fig. 29 A). All cells that were positive for eGFP, BrdU and NeuN were regarded as graft-derived neurons whereas all cells that were positive for BrdU and NeuN but negative for eGFP constituted endogenous host-derived newborn neurons that were generated after implantation of TNR⁺ or TNR⁻ cells.

As expected, the number of graft-derived eGFP⁺/BrdU⁺/NeuN⁺ cells in TNR⁺ grafts was significantly higher compared to TNR⁻ grafts (fig. 29 B). Grafts consisting of TNR⁺ cells contained 4.5 ± 0.5 eGFP⁺/BrdU⁺/NeuN⁺ cells per 40x visual field whereas in TNR⁻ grafts only 2 ± 0.5 eGFP⁺/BrdU⁺/NeuN⁺ cells could be identified per 40x visual field. These data confirm the finding that tenascin-R enhances neuronal differentiation of ESC-derived cells in vivo.

Next, the number of eGFP⁻/BrdU⁺/NeuN⁺ host-derived newborn neurons was counted in mice that had been injected with TNR⁺ cells, TNR⁻ cells or PBS (fig. 29 C). In mice that had received a QA-lesion and an injection with PBS no host-derived newborn neurons could be identified in the striatum. In contrast, engrafted TNR⁺ and TNR⁻ cells induced the generation of eGFP⁻/BrdU⁺/NeuN⁺ host-derived newborn neurons in the striatum. In grafts consisting of TNR⁺ cells 9 ± 2 eGFP⁻/BrdU⁺/NeuN⁺ host-derived newborn neurons were counted per 40x visual field. This number was significantly higher compared to the number of eGFP⁺/BrdU⁺/NeuN⁺ cells in TNR⁻ grafts (3 ± 1 host-derived newborn neurons).

These data show that tenascin-R enhanced the number of newly generated host-derived postmitotic neurons within the grafted area. Importantly, no newly generated endogenous postmitotic neurons were observed in PBS-injected QA-lesioned animals.
Results

Figure 29: Tenascin-R enhances the number of host-derived newly generated neurons within the grafted area. QA-lesioned mice were labeled with BrdU from day 3 to day 7 days after transplantation of TNR⁺ and TNR⁻ cells or after sham injection. (A) Laser scanning microscopy illustrates immuno-histochemical analysis of TNR⁺ and TNR⁻ grafts and of the PBS-injected lesion with BrdU (red) and the neuronal marker NeuN (blue) 1 month after transplantation of eGFP⁺ (green) cells. Graft-derived newborn neurons (eGFP⁺/BrdU⁺/NeuN⁺) in merged images appear white whereas host-derived newborn neurons (eGFP⁻/BrdU⁺/NeuN⁺) appear purple in merged pictures. Note the enhanced number of both donor-derived and host-derived newborn neurons in TNR⁺ grafts and the absence of any newborn neurons in the PBS injected lesioned striatum. Scale bar, 100µm. (B) Quantification of graft-derived newborn neurons (eGFP⁺/BrdU⁺/NeuN⁺) and (C) quantification of host-derived newborn neurons (eGFP⁻/BrdU⁺/NeuN⁺) showed enhanced numbers of both, graft-derived and host-derived newborn neurons within TNR⁺ versus TNR⁻ grafts. The graphs show mean values ± s.e.m. Student’s t test was performed for statistical analysis (*p: 0.01).
4.9 Effects of engrafted TNR⁺ and TNR⁻ cells on the behavioural performance of QA-lesioned mice

Patients suffering from Huntington’s disease show impaired motor functions including impairment of balance with disturbed gait control, hypo- or hypertonia of muscles and typically hyperkinetic movements of the trunk and extremities leading to a state called chorea after prolonged duration of the disease.

Since the right striato-thalamo-cortical circuitry was disrupted in mice that had been intrastriatally injected with QA, it was interesting to analyze if motor functions were impaired in these mice. Moreover, it should be analyzed if the engraftment of tenascin-R secreting or non-secreting cells could promote functional recovery of these animals.

Since QA-lesioned animals did not show any obvious motor deficits such as hyperkinesia, ataxia, tremor or disabilities in walking, the following behavioural tests were performed to unravel an impairment of motor functions in these mice.

4.9.1 QA-lesioned animals did not show an impairment of motor functions in the Rotarod test

The Rotarod test is a suitable method to test if mice have deficits in motor coordination or an impairment of balance. Unlesioned mice and mice that received a QA-lesion one week before testing were analyzed in a Rotarod test. The latency to fall off the rotating beam was measured three times with an accelerating rotation of the beam. As shown in figure 30, no differences between these 2 groups could be observed showing that the unilateral lesion did not affect motor coordination and balance control of QA-lesioned animals in this test.
Results

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Figure 30: Rotarod-test on QA-lesioned animals. An accelerated Rotarod-test was performed with three trials on non-lesioned animals and on mice that have been lesioned with QA one week before. Both groups showed a similar latency to fall indicating that motor coordination was not affected after lesion in this test. The graph shows mean values ± s.e.m.

4.9.2 Engrafted TNR⁺ and TNR⁻ cells did not influence the rotation behaviour of QA-lesioned mice after intraperitoneal injection of apomorphine

Due to the unilateral destruction of the striato-thalamo-cortical circuitry QA-lesioned mice rotated to the ipsilateral side in the open field when intraperitoneally injected with the dopamine agonist apomorphine. According to the transplantation paradigm mice received an engraftment of TNR⁺ or TNR⁻ cells or an injection of PBS 3 days after lesion. Rotation was measured 2, 8, 21, 31 and 45 days after lesion in the open field (fig. 31). Mice were excluded that showed a rotation bias less than 2 degrees/cm distance moved. Functionally lesioned mice were equally distributed among the three groups before transplantation.

Unexpectedly, no differences in the rotation bias could be observed between mice that received TNR⁺ or TNR⁻ cells or an injection with PBS in the test period from 8 to 45 days. In all groups there was a slight but not significant decrease of ipsilateral rotations 8 to 45 days after lesion.
These data indicate that despite conclusive histological findings neither the engraftment of stem cells nor an enhanced secretion of tenascin-R by these stem cells had a beneficial effect on the functional performance of QA-lesioned mice compared to a sham injection with PBS.

**Figure 31:** Analysis of apomorphine-induced rotations in animals with unilateral QA-induced striatal lesion grafted with pre-differentiated TNR⁺ cells, TNR⁻ cells or sham-injected with PBS. Relative meander was calculated as turning angle divided by distance moved. Mean values ± s.e.m. are shown. No differences in ipsilateral rotation were observed between animals grafted with TNR⁺ cells, TNR⁻ cells and sham-injected controls.
5. Discussion

This study aimed to unravel the effects of the extracellular matrix molecule tenascin-R on several aspects of ESC-mediated neuroregeneration in an excitotoxic mouse model of Huntington’s disease. To that end, effects were analyzed that included neural differentiation of murine ESCs \textit{in vitro} and their further maturation \textit{in vivo}, migration of differentiated stem cells \textit{in vitro} and \textit{in vivo}, generation of synaptic connections between engrafted cells and host-derived neurons, generation of host-derived newborn neurons and their migration after intrastriatal transplantation of ESC-derived cells and finally, behavioural performance of quinolinic acid (QA)-lesioned animals after treatment with pre-differentiated TNR$^+$ and TNR$^-$ ESCs.

In comparison to TNR$^-$ ESCs, TNR$^+$ cells showed enhanced differentiation into postmitotic neurons and a tendency towards reduced differentiation into astrocytes \textit{in vitro}. One and 2 months after transplantation, tenascin-R promoted neuronal differentiation at the expense of astrocytes and increased the generation of GABAergic neurons. Migration of pre-differentiated ESCs was reduced by tenascin-R in a scratch assay \textit{in vitro} and also reduced \textit{in vivo}. In contrast, lateral migration of endogenous neuroblasts in the rostral migratory stream and radial migration of these cells was significantly enhanced by implanted stem cells secreting tenascin-R. Indeed, tenascin-R increased detachment of migrating neuroblasts from the rostral migratory stream and their attraction towards engrafted stem cells. Furthermore, tenascin-R promoted the recruitment of endogenous newborn neurons in the grafted area. However, a beneficial effect of implanted TNR$^+$ and TNR$^-$ cells on functional recovery of QA-lesioned mice was not observed up to 42 days after transplantation.

5.1 Tenascin-R promotes neuronal differentiation of murine ESCs \textit{in vitro} and after transplantation into QA-lesioned mice

Huntington’s disease is associated with a loss of striatal GABAergic medium-sized projection neurons constituting about 90% of all striatal neurons (Gerfen et al., 1990). Thus, a striatal cell replacement approach with \textit{in vitro}-expanded neural cells might be a helpful strategy to replace degenerated GABAergic neurons and to re-establish damaged neuronal circuitries within the basal ganglia. In this study, murine ESCs were genetically modified to overexpress and secrete the extracellular matrix molecule tenascin-R \textit{in vitro} and also \textit{in vivo}. This
molecule was chosen because tenasin-R is expressed by a subset of neuronal cells in the CNS, has been shown to influence neurite outgrowth and migration of neural cells in vitro (for review Jones and Jones, 2000), to promote the generation of inhibitory synapses in vivo (Nikonenko et al., 2003) and to be essential for the maturation of particular inhibitory interneurons in the motor and sensory cortex (A. Irinchev, unpublished observations). Moreover, tenasin-R directs migration of host-derived neuroblasts towards the site of enhanced expression in the non-lesioned adult forebrain (Sagathelyan et al., 2004) and mediates neuroprotection of motorneurons in the brainstem after traumatic facial nerve injury as a component of perineuronal nets (Angelov et al., 1998). Therefore, tenasin-R is a promising candidate molecule for stem cell-mediated neuroregeneration in the CNS.

TNR⁺ and TNR⁻ ESCs were differentiated according to a modified lineage selection protocol (Okabe et al., 1996; Lee et al., 2000) that yields a high number of neural precursor cells in the presence of FGF-2 (stage 4). By withdrawal of FGF-2 differentiation into postmitotic neurons, astrocytes and oligodendrocytes was efficiently induced (stage 5). In this study, tenasin-R was abundantly expressed by TNR⁺ ESCs but not by TNR⁻ ESCs throughout differentiation in vitro at stages 1, 4 and 5 and also in vivo up to two months after transplantation into QA-lesioned animals. Tenasin-R expression was driven by the PGK-(phosphoglyceratkinase) promoter that is known to be active in undifferentiated ESCs and also in ESC-derived neural cells after transplantation in the QA mouse model of HD (Bernreuther et al., 2006). Tenasin-R was expressed in different glyco- and isoforms in vitro and in vivo. Undifferentiated TNR⁺ ESCs (stage 1) showed a single band of tenasin-R with a molecular weight of around 220 kDa in Western blot analysis whereas two bands at 160 kDa (tenasin-R 160) and 180 kDa (tenasin-R 180) were detected in differentiating (stage 4) and differentiated (stage 5) TNR⁺ ESCs and also in engrafted TNR⁺ cells. These two isoforms are typically found in the adult brain and have been described to be spatially and temporally regulated in different brain areas (Pesheva et al., 1989). Tenasin-R 160 lacks a portion of its aminoterminal region and is only weakly expressed right after birth but is the predominant isoform in adulthood. In contrast, tenasin-R 180 is the first isoform to be found after birth but its expression declines during postnatal life (Pesheva et al., 1989; Woodworth et al., 2004). As seen in this study, the tenasin-R 180 isoform was more strongly expressed by TNR⁺ ESC-derived cells at stage 4, at stage 5 and also after transplantation when compared to the expression of tenasin-R 160, pointing towards a regulated expression of tenasin-R isoforms also in these cells.
Tenascin-R is modified by posttranslational N- and O-glycosylation (Pesheva et al., 1989; Probstmeier et al., 2000). Besides O-linked chondroitine sulfate glycosaminoglycans, N-linked carbohydrate residues have been identified on tenascin-R that contain the human natural killer antigen-1 (HNK-1) and other sulfated sugar residues (Woodworth et al., 2002 and 2004). PNGase F digestion of tenascin-R secreted by undifferentiated TNR+ ESCs revealed that the high molecular form of 220 kDa carried a high amount of N-linked carbohydrate residues. Such a glycosylation pattern has also been demonstrated for PNGase F-treated tenascin-R protein produced by the human neuroblastoma cell line SH-SY5Y in vitro (Woodworth et al., 2004). In contrast, the tenascin-R 160 and tenascin-R 180 isoforms appear to be less N-glycosylated (Pesheva et al., 1989). These data indicate that the expression of glycosyltransferases differs in undifferentiated TNR+ ESCs from that in differentiating TNR+ cells at stage 4, differentiated TNR+ cells at stage 5 and also from that in engrafted TNR+ cells in vivo.

Interestingly, a cell-type-specific sulfatation of tenascin-R has been described in the postnatal brain where neurons and oligodendrocytes secrete the two tenascin-R isoforms with different sulfated carbohydrate residues due to differential expression of sulfotransferases (Woodworth et al., 2004). A precise characterization of carbohydrate epitopes on tenascin-R isoforms in combination with an analysis of stage- and cell-type-specific expression of different glycosyl- and sulfotransferases during in vitro-differentiation of ESCs remains to be studied in more detail.

One important aim of this study was to unravel the effect of tenascin-R on neuronal differentiation of murine ESCs in vitro and after transplantation in the QA mouse model of HD. Since a degeneration of striatal inhibitory GABAergic neurons occurred after excitotoxic lesion of the striatum in these mice, one major goal was to further enhance the total number of donor-derived GABAergic neurons by tenascin-R after intrastriatal transplantation of pre-differentiated ESCs.

Tenascin-R did not influence the differentiation of ESCs into nestin+ neural precursor cells in vitro (stage 4) and did not alter proliferation and apoptosis of differentiating cells. In contrast, tenascin-R significantly enhanced in vitro-differentiation of neural precursor cells into postmitotic neurons (33% of all TNR+ cells versus 26% of all TNR- cells) whereas glial differentiation was not significantly altered by tenascin-R with a tendency towards decreased astrocytic differentiation.

The striatum is regarded as non-neurogenic tissue that does not favour neuronal differentiation of implanted cells. Indeed, studies on intrastriatal implantation of E.14 fetal
neural progenitor cells demonstrated that the majority of cells differentiated into astrocytic cells whereas only poor neuronal and oligodendrocytic differentiation could be observed in grafts (Johann et al., 2007). In contrast, prominent neuronal differentiation of ESC-derived cells was observed after transplantation into the QA-lesioned striatum. Pre-differentiated TNR⁺ cells showed an approximately twofold increase in neuronal differentiation but reduced astrocytic and unaltered oligodendrocytic differentiation after transplantation when compared to TNR⁻ cells. One and 2 months after implantation TNR⁺ grafts contained about 39,000 and 38,500 neurons (corresponding to 40% and 31% of all implanted TNR⁺ cells) whereas TNR⁻ grafts contained approximately 15,500 and 17,000 neurons (corresponding to 23% and 15% of all implanted TNR⁻ cells) 1 and 2 months after engraftment, respectively. Interestingly, tenascin-R did not influence relative GABAergic, dopaminergic and also cholinergic subtype specification of neurons. In vitro, about 80% of all TNR⁺ and TNR⁻ neurons acquired a GABAergic phenotype and in vivo, about 50% of all neurons in TNR⁺ and TNR⁻ grafts showed a GABAergic specification. However, tenascin-R favoured neuronal differentiation and thus, the total number of donor-derived GABAergic neurons was significantly enhanced in TNR⁺ grafts (about 20,500 GAD⁺ neurons 1 month and 23,000 GAD⁺ neurons 2 months after transplantation). In comparison, only 7,000 and 14,000 donor-derived GAD⁺ GABAergic neurons were observed in TNR⁻ grafts at these two time points. These data indicate that tenascin-R is a beneficial molecule in the context of striatal regeneration since it leads to an increased total number of GABAergic neurons in vivo, the cell type lost in the course of HD.

Tenascin-R belongs to the group of extracellular matrix molecules that are known to direct the differentiation of cells in vitro and in vivo via several mechanisms (for review see Adams and Watt, 1993). They bind to extracellular matrix receptors on differentiating cells including integrin and non-integrin receptors, thus mediating cell adhesion and activation of intracellular signal cascades. Since the expression of integrins on cells can differ during differentiation - as has been shown for murine ESCs in vitro (Cooper et al., 1991) - different extracellular matrix proteins may influence the differentiation of a certain subset of cells. On the other hand, the presentation of the same extracellular matrix protein in different splice and glycosylated forms can alter cell binding properties and communication with other matrix molecules, as shown for the binding of tenascin splice variants to fibronectin (Chiquet-Ehrismann et al., 1991), thereby changing environmental cues for differentiating and matrix-binding cells. Different domains of tenascin-R and their carbohydrate epitopes have been demonstrated to bind to surface molecules on neurons (for review see Vaughan et al., 1994).
Tenascin-R-binding membrane proteins include contactin/F3 (Brümmendorf et al., 1989), the heparan sulfate proteoglycan glypican (Vaughan et al., 1994), GABA$_\text{R}$ receptors (Saghatel\-yan et al., 2000), the $\beta$-subunit of voltage-gated sodium channels (Srinivasan et al., 1998) and sulfoglucuronyl carbohydrate binding protein -1 (SBP-1; Chou et al., 2000). Furthermore, neurite outgrowth experiments revealed that different domains of tenascin-R promote or inhibit the outgrowth of axons and dendrites, suggesting a more complex regulation of the interaction between tenascin-R and neurons \textit{in vitro}. In this context, it has been shown that the $11^\text{th}$ fibronectin (FN) type III homologues repeat domain of tenascin-R induces neurite extension of cerebellar (Husmann et al., 1992), hippocampal and mesencephalic neurons (Lochter et al., 1991) whereas the epidermal growth factor (EGF)-like repeats and the cysteinn-rich NH2 terminal stretch of tenascin-R reduces the outgrowth of hippocampal neuronal processes \textit{in vitro} (Xiao et al., 1997).

The stimulating effect of tenascin-R on neuronal differentiation of ESC-derived neural precursor cells could be mediated by direct interaction of neuronal surface molecules and different domains of tenascin-R secreted by TNR$^+$ cells. On the other hand, enhanced secretion of tenascin-R into the extracellular space changes the composition of the extracellular matrix providing a new scaffold for differentiating cells. Importantly, extracellular matrix proteins have been demonstrated to bind and to concentrate growth and neurotrophic factors resulting in a prolonged activity of these beneficial molecules (Nathan and Sporn, 1991). Indeed, matrix-bound fibroblast growth factor (FGF) is degraded more slowly than free FGF leading to a prolonged activity of this growth factor (Klagsbrun, 1990). It has been demonstrated recently that murine ESCs have the intrinsic ability to express and secrete neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophic factor (NT)-3 (Bentz et al., 2007). Thus, abundant tenascin-R might help to concentrate and to present these factors to differentiating TNR$^+$-cells over a prolonged period of time \textit{in vitro} and \textit{in vivo} although direct binding of tenascin-R to BDNF has not been described so far. Additionally, it has been demonstrated recently that different domains of tenascin-R activate microglia to secrete neurotrophic factors such as NGF and BDNF (Liao et al., 2005). While secretion of NGF is triggered by the FN-domains 6 - 8, BDNF secretion was activated by binding of the EGF-like repeats of tenascin-R to microglial cells. As shown in this study, activated microglia was abundantly present in the QA-lesioned striatum and thus, tenascin-R secreted by implanted TNR$^+$ cells might modulate microglial reaction leading to enhanced secretion of these neurotrophic factors.
It is important to note that BDNF enhances neurogenesis in the unlesioned and in the QA-lesioned adult brain when overexpressed in the subventricular zone by recombinant adeno-associated virus delivery (Henry et al., 2007). Furthermore, BDNF was proposed to be important for the maturation of GABAergic medium-sized spiny projection neurons \textit{in vitro} and \textit{in vivo} (Ivkovic and Ehrlich, 1999). In this context, it should be emphasized that an impaired axonal transport of BDNF along cortical projection neurons towards striatal medium-sized projection neurons contributes to the lack of BDNF in the striatum of HD patients (Gauthier et al., 2004). This lack of BDNF in turn causes a disturbed development and survival of these striatal GABAergic projection neurons that are important for the modulation of voluntary movements (Bemelmans et al., 1999). Thus, BDNF might on the one hand contribute to neuronal differentiation of cells in TNR\(^+\) grafts and might on the other hand even prevent endogenous host-derived striatal neurons from dying. Indeed, BDNF has been demonstrated to reduce excitotoxicity-induced death of medium-sized GABAergic neurons when continuously infused into the striatum in a rat model of mild ischemic brain injury (Galvin and Oorschot, 2003).

Several protocols exist to direct the differentiation of murine ESCs into neuronal cell types in order to apply these cells in different animal models of neurological diseases (Bain et al., 1995; Okabe et al., 1996; Kawasaki et al., 2000; Lee et al., 2000; Tropepe et al., 2001). The lineage selection protocol was used in this study because it provides the opportunity to propagate and thus, to efficiently enhance the number of neural precursor cells at later stages of differentiation (stage 4). Interestingly, these neural precursor cells start to form neural aggregates when cultured over a prolonged period of time in the presence of FGF-2 (Dihné et al., 2006). These aggregates were termed SENAs (substrate-adherent embryonic stem cell-derived neural aggregates) and contain a high amount of neuronally committed cells that migrate from the outer part of these aggregates towards the periphery. More than 90\% of cells within SENAs become neurons when inducing terminal differentiation by withdrawal of FGF-2 (Dihné et al., 2006). Both TNR\(^+\) and TNR\(^-\) ESCs differentiated into SENAs (data not shown) but their structure appeared to be altered in the presence of tenascin-R characterized by disturbed migration of TNR\(^+\) cells from the outer part of SENAs towards the periphery. This observation is in line with the fact that TNR\(^+\) cells migrated less in a scratch assay \textit{in vitro}. Indeed, this assay showed that migration of ESC-derived cells is inhibited by tenascin-R and demonstrated that this effect is dependent on the amount of tenascin-R presented to migrating cells. Both promoting and inhibiting effects of tenascin-R on the migration of cells have been demonstrated \textit{in vitro}. Substrate bound-tenascin-R enhances the migration of
cerebellar granule cells (Husmann et al., 1992) and neuroblastoma cells (Halfter et al., 1989). In contrast, tenascin-R reduces the migration of neural cells when presented in soluble form to these cells (Halfter et al., 1989). In this study, ESC-derived TNR⁺ neural cells secreted tenascin-R into the culture medium and thus, tenascin-R was presented as a soluble molecule to differentiating cells. Therefore, similar mechanisms for cellular repulsion involving the FN type III homologues repeats of tenascin-R (Haftet al., 1989) might also account for the reduced migration of ESC-derived cells by soluble tenascin-R in vitro.

5.2 Tenascin-R influences several aspects of graft-host interaction in stem cell-treated QA-lesioned mice

With regard to a cell replacement strategy for HD, the migration of implanted cells into the host tissue is an important parameter for the integration of engrafted cells favouring a higher degree of functional connectivity to basal ganglia circuitries (for review see Dunnett and Rosser, 2007). As shown in this study, in vitro-labeled and intrastriatally implanted ESC-derived cells performed very poor migration into the host striatum as visualized by MRI scans about 1 month after engraftment of cells. In contrast, intrastriatally implanted fetal neural progenitors are known to migrate intensively within the host striatum and have been shown to enter adjacent brain areas or even to migrate into the contralateral hemisphere (Englund et al., 2002). It remains questionable if such intensive migration is favourable for regenerating striatal tissue since implanted cells should exert their functions within the lesioned striatum (Dunnett and Rosser, 2007). But poor migration of engrafted ESC-derived cells in the recipient brain remains a problem in the context of intrastriatal integration of cells. Thus, the effect of tenascin-R on migration in vivo was analyzed in more detail. Interestingly, migration of implanted TNR⁺ cells into the QA-lesioned striatum was slightly but significantly reduced 1 and 2 months after transplantation in comparison to the migration of TNR⁻ cells. While TNR⁻ cells migrated a distance of 82µm and 71µm into the host striatum 1 month and 2 months after transplantation, TNR⁺ cells migrated about 20µm less at both time points. Therefore, an inhibitory effect of tenascin-R on the migration of stem cells was also observed in vivo and has to be regarded as a non-beneficial effect of tenascin-R on stem cell-mediated regeneration. Tenascin-R has been shown to serve as a detachment signal for migrating cells in the olfactory bulb (Sagathelyan et al., 2004) and in the developing cerebellar cortex (Husmann et al., 1992). Interestingly, both attractant (Sagathelyan et al., 2004) and repulsive (Husmann et al., 1992) guidance properties of tenascin-R were described.
Discussion

for migrating cells in vivo. Furthermore, in vitro-studies suggested that - as discussed for other parameters - tenasin-R has both promoting (Xiao et al., 1996) and inhibiting (Lochter et al., 1991; Xiao et al., 1996) functions on cellular adhesion depending on the interaction between cells and different domains of the tenasin-R molecule. It is unlikely that reduced migration of TNR+ cells was due to mechanical inhibition since secreted tenasin-R did not form a scar-like boundary around intrastral grafts. Interestingly, tenasin-R mediates the attachment of neurons to astrocytes in binding assays in vitro (Kruse et al., 1995, Grumet et al., 1985). Such neuron-glia interaction could explain the altered migration of implanted TNR+ cells in vivo. Indeed, TNR+ and TNR- grafts contain ESC-derived astrocytes that are located in the center but also at the periphery of the grafts and therefore, tenasin-R could mediate reduced migration of donor-derived cells by enhancing binding to these donor-derived astrocytes. A further very important parameter for the integration of implanted cells in the recipient brain constitutes the synaptic connectivity between striatal host neurons and graft-derived cells. Two different aspects of synaptogenesis were analyzed at the interface between the host striatum and the stem cell grafts. Both synaptic input from endogenous host-derived neurons to engrafted cells and synaptic output from engrafted cells to host-derived neurons were measured 1 month after transplantation. 6.7% of all TNR+ cells and 4.4% of all TNR- cells were covered with SV-2+ host-derived synaptic boutons and 2.7% all TNR+ cells and 1.5% of all TNR- cells had axons covering host neurons with SV-2+ synaptic terminals. These results showed a tendency for tenasin-R to enhance afferent and efferent synaptic connection between donor-derived and host-derived neurons although these observed differences were not statistically significant. Studies on TNR-deficient animals revealed more clearly an important role of tenasin-R on synaptogenesis (for review see Dityatev and Schachner, 2003). TNR-deficient mice show impaired synaptic transmission and impaired synaptic plasticity in the CA1 region of the hippocampus (Saghatelyan et al., 2000 and 2001). Interestingly, perisomatic synapses on inhibitory GABAergic interneurons in the CA1 pyramidal cell layer are reduced (Nikonenko et al., 2003) and perineuronal nets surrounding these neurons show structural abnormalities in these animals (Brückner et al., 2000). Furthermore, motorneurons in the spinal cord of TNR- deficient mice show reduced coverage with excitatory and inhibitory perisomatic synaptic boutons suggesting that tenasin-R is important for synaptogenesis in vivo (Apostolova et al., 2006). As seen in the present study, the formation of synaptic connections between host and grafted cells occurs at a low level in both the TNR+ and the TNR- group. However, these results show
that engrafted ESC-derived neurons communicate with the host tissue as has been
demonstrated for ESC-derived cells implanted in an animal model of stroke (Bühnemann et
al., 2006) or in an animal model of Parkinson’s disease (Kim et al., 2002).

In this study, special emphasis was put on evaluating the effects of tenasin-R on the
generation and migration of host-derived neuroblasts in the adult brain after QA-lesion and
implantation of ESC-derived neural cells. In the adult unlesioned brain of rodents and
primates newborn neurons are generated in neurogenic areas including the SVZ of the lateral
ventricles and the subgranular layer of the dentate gyrus in the hippocampus (for review see
Sohur et al., 2007). While cells in the hippocampus perform only poor migration, neural
progenitors in the anterior forebrain arise from the SVZ and migrate tangentially along the
rostral migratory stream (RMS) to the olfactory bulb where they differentiate into olfactory
granule and peri-glomerular interneurons (Luskin 1993; Lois and Alvarez-Buylla 1994;
Betarbet et al., 1996; Winner et al., 2002). Although it has been intensively discussed if such
migration also occurs in humans (Sanai et al., 2004) evidence for the existence of migrating
cells in a rudimental RMS in humans has been demonstrated recently (Curtis et al., 2007). In
several animal models progenitor cells within the SVZ are activated to proliferate and to
migrate towards lesioned areas as has been demonstrated for epileptic (Parent et al., 2002a),
ischemic (Nakatomi et al., 2002; Parent et al., 2002b), physical (Magavi et al., 2000) and
excitotoxic (Tattersfield et al., 2004) lesions of hippocampal, cortical and striatal tissue.

Interestingly, a unilateral striatal QA-lesion in rats leads to enhanced proliferation of cells in
the ipsilateral SVZ and to enhanced migration of neural progenitor cells from the SVZ
directly into the adjacent striatum (Tattersfield et al., 2004; Gordon et al., 2007). Furthermore,
about 20% of these migrating cells acquired a neuronal phenotype in the QA-lesioned
striatum (Tattersfield et al., 2004). Interestingly, a recruitment of residing neural precursor
cells has also been found in brains of Huntington patients as demonstrated by an increased
cell proliferation and subsequent neurogenesis in subcortical areas (Curtis et al., 2003).

These findings raised the question if the implantation of pre-differentiated stem cells and the
presence of tenasin-R might enhance neurogenesis in the SVZ and might promote
recruitment of host-derived neuroblasts from the SVZ and from the RMS. Such phenomenon
would constitute an important aspect in the context of a stem cell-mediated striatal
neuroregeneration in QA-lesioned animals. For that, a third group of mice was included that
received a sham injection with PBS instead of stem cells 3 days after lesion.

In line with previous observations, an enhanced size of the ipsilateral SVZ could be observed
in mice after unilateral striatal lesion with QA. Furthermore, migration of host-derived
doublecortin\textsuperscript{+} neural progenitor cells directly into the adjacent striatum was seen 1 and also 2 months after lesion in the TNR\textsuperscript{+}, TNR\textsuperscript{−} and also in the PBS group. Since no differences between the groups could be observed, these effects were induced by the lesion rather than by the implantation of stem cells. Indeed, reactive microglia cells that are present in the lesioned striatum have been proposed to attract host-derived neuroblasts towards lesioned brain areas by secreting attractant factors including stromal cell-derived factor-1\textalpha (SDF-1\textalpha) and monocyte chemoattractant protein-1 (MCP-1; Aarum et al., 2003; Guillemin et al., 2003). TNR\textsuperscript{+} and TNR\textsuperscript{−} grafts were located in the dorsolateral part of the striatum with a mean distance from the SVZ of more than 500\textmu m in both groups that may explain the lack of a stem-cell effect in this context.

It was shown in this study that TNR\textsuperscript{+} and TNR\textsuperscript{−} cells influenced tangential migration of endogenous host-derived neuroblasts in the posterior extension of the RMS. In contrast to the PBS control, both TNR\textsuperscript{+} and TNR\textsuperscript{−} cells enhanced migration of doublecortin\textsuperscript{+} cells from the RMS to the lateral areas of the brain between the corpus callosum and the dorsal margin of the lesioned striatum. Interestingly, this attracting effect was accelerated by the presence of tenascin-R. Furthermore, it was shown that in contrast to the PBS control TNR\textsuperscript{+} and TNR\textsuperscript{−} grafts induced the detachment of doublecortin\textsuperscript{+} cells from the RMS as these host-derived neuroblasts migrated radially through the striatum towards the engrafted TNR\textsuperscript{+} and TNR\textsuperscript{−} stem cells. Importantly, this attraction of host-derived neuroblasts was enhanced in the TNR\textsuperscript{+} group up to 2 months after transplantation suggesting that tenascin-R sustainedly stimulated the recruitment of migrating endogenous neuroblasts from the RMS.

In line with this, studies on TNR-deficient mice revealed that tenascin-R mediates the detachment of migrating neuroblasts from the RMS and favours radial migration in the olfactory bulb (Saghatelyan et al., 2004). Indeed, migrating cells from the RMS do not properly enter their cortical target area in TNR-deficient animals but are found to be stuck in the olfactory RMS (Weber et al., 1999; Saghathelyan et al., 2004). Furthermore, attractant guidance properties of tenascin-R have been demonstrated in another paradigm. It has been shown that tenascin-R-secreting BHK cells could attract residing and migrating neuroblasts from the SVZ and the RMS when implanted into the most anterior part of the striatum or into the cerebral cortex in close proximity to the RMS (Saghatelyan et al., 2004). Importantly, these residing and migrating cells were not attracted by implanted non-transfected cells pointing out that tenascin-R was the molecule that recruited endogenous neural precursor cells from neurogenic towards non-neurogenic sites in the brain possibly due to direct cell-matrix-interaction.
As seen in this study, TNR\textsuperscript{+} but also TNR\textsuperscript{-} cells attracted host-derived neural precursor cells from the RMS suggesting that implanted ESC-derived cells secrete additional beneficial guidance molecules that show an effect on migrating cells. Interestingly, astrocytes have been suggested to regulate the migration of SVZ progenitor cells by secreting an astrocyte-derived migration-inducing activity factor (Mason et al., 2001). Since astrocytic cells are present in both TNR\textsuperscript{+} and TNR\textsuperscript{-} grafts, this factor might be involved in attracting migrating cells towards the engrafted striatum. Other putative attractant guidance molecules include neurotrophic factors such as BDNF, NGF and NT-3 that are expressed by differentiating ESC-derived cells (Bentz et al., 2007). Indeed, intraventricular application of BDNF enhanced neurogenesis in the SVZ (Zigova et al., 1998) and induced migration of neural precursor cells from the SVZ into non-neurogenic areas including the septum, thalamus, hypothalamus and the striatum (Benraiss et al., 2001; Pencea et al., 2001).

Doublecortin\textsuperscript{+} cells were detected within the QA-lesioned striatum of stem cell-treated but not of sham-injected mice. Furthermore, BrdU\textsuperscript{+} and NeuN\textsuperscript{+} host-derived newborn neurons were observed within TNR\textsuperscript{+} and TNR\textsuperscript{-} grafts but not in the QA-lesioned and PBS-injected striatum. These results indicate that implanted pre-differentiated stem cells induce the recruitment of host-derived newborn neurons in the QA-lesioned striatum. It remains an open question if these cells have been recruited from the RMS or if these cells have been generated \textit{in situ} within the lesion-site. Indeed, neural precursor cells have been found in the striatum that can be propagated and differentiated into astrocytes, oligodendrocytes and neurons \textit{in vitro} (Palmer et al., 1995) underlining the possibility of an \textit{in situ}-neurogenesis within the adult striatum. Interestingly, tenascin-R enhanced the number of newly generated postmitotic neurons within the lesioned striatum. This is in line with the observation that tenascin-R promotes neuronal maturation of ESC-derived neural precursor cells \textit{in vitro} and \textit{in vivo}.

Taken together, these data show that tenascin-R promotes the stem cell-mediated recruitment of host-derived neural precursor cells in a mouse model of HD.
5.3 Engrafted TNR<sup>+</sup> and TNR<sup>-</sup> stem cells do not promote functional recovery of QA-lesioned mice

This study demonstrated that tenasin-R influences several aspects of ESC-mediated neuroregeneration in the QA-model of HD. However, neither TNR<sup>+</sup> nor TNR<sup>-</sup> cells could improve the behavioural performance of QA-lesioned mice as judged by measuring rotational asymmetry in comparison to PBS-injected QA-lesioned animals.

In contrast, several studies on different animal models of HD showed that implantation of different cell sources can lead to an improvement of functional impairment in these animals. These cell sources included primary fetal striatal grafts, cells from the fetal and adult SVZ, non-neural cells and also pre-differentiated ESCs (for review see Dunnett and Rosser, 2007). These studies emphasized that a cell replacement strategy with neural cells might indeed be a suitable approach to address HD in humans. Therefore, clinical trials have been established using primary fetal tissue for transplantation in HD patients with variable outcomes (for review see Dunnett and Rosser, 2004).

Intrastriatal transplantation and behavioural studies on QA-lesioned animals were predominantly performed on rats. Effects on apomorphine-induced rotational asymmetry was tested after implantation of a variety of cell sources including fetal neural progenitor cells (Visnyei et al., 2006), neural progenitor cells from the adult SVZ (Vazey et al., 2006), an immortalized cells line (Lee et al., 2005), encapsulated BHK cells secreting human ciliary-derived neurotrophic factor (hCNTF; Emerich et al., 1996) and murine pre-differentiated ESCs overexpressing the neuronal surface molecule L1 (Bernreuther et al., 2006). Interestingly, all cell types - except for fetal neural progenitor cells (Visnyei et al., 2006) - reduced QA-induced rotational asymmetry although some grafts were devoid of GABAergic neurons (Lee et al., 2005) or of any neurons (Emerich et al., 1996) suggesting that functional recovery was caused by neuroprotection of endogenous cells rather than by replacement of degenerated neurons in these two studies. On the other hand, grafts consisting of differentiated adult neural progenitor SVZ cells (Vazey et al., 2006) and of differentiated L1-overexpressing ESCs (Bernreuther et al., 2006) contained donor-derived GABAergic neurons causing functional recovery in these QA-lesioned animals.

The lack of functional recovery in QA-lesioned mice after implantation of TNR<sup>+</sup> and TNR<sup>-</sup> cells might be explained by different aspects. On the one hand, reduced or normal migratory behaviour of TNR<sup>+</sup> and TNR<sup>-</sup> cells, respectively, might not be sufficient to re-populate enough territory in the lesioned striatum to contact target areas of striatal GABAergic projection neurons in the basal ganglia and therefore to promote functional recovery. On the
other hand, communication between implanted ESC-derived cells and host-derived neurons could be observed in both, the TNR⁺ and the TNR⁻ group. However, the degree of synaptic interaction was only moderate and fibre association between graft-derived and host-derived neurons within the basal ganglia might not have been correct accounting for the lack of a functional effect in QA-lesioned mice.

It is possible, that the total number of GABAergic cells within the ESC-derived grafts might be insufficient to elicit beneficial effects in animals although more than 20,000 TNR⁺ and more than 7,000 TNR⁻ GAD⁺ GABAergic neurons were observed within the grafts in the QA-lesioned striatum. However, it should be mentioned that less than 1,000 GABAergic neurons derived from the adult SVZ were sufficient to reduce apomorphin-induced rotational asymmetry (Vazey et al., 2006). These differences might raise the question if ESCs have the intrinsic ability to precisely differentiate into neurons with a real striatal-like GABAergic neuronal phenotype characterized by the expression of GAD, DARPP-32, STEP (striatal enriched phosphates), enkephalin, substance P, dynorphin and calbindin as these neurons have to be replaced in QA-lesioned animals (Dunnett and Rosser, 2007). However, two independent studies demonstrated that intrastriatally implanted pre-differentiated primate and murine ESCs differentiated into striatal-like GABAergic neurons expressing DARPP-32 and calbindin (Bühnemann et al., 2006; Hayashi et al., 2006). Importantly, in both studies about 5-6% of all implanted cells acquired this specific phenotype of striatal-like GABAergic neurons. It remains open if this degree of differentiation is sufficient to replace degenerated striatal neurons but it is comparable with the degree of striatal subtype specification of adult SVZ-derived neurons after engraftment (Vazey et al., 2006). These findings favour the assumption that insufficient communication between host-derived and ESC-derived neurons accounts for a lack of functional recovery in this study rather than insufficient differentiation of implanted TNR⁺ and TNR⁻ cells into a specific neuronal subtype.

Interestingly, adult SVZ-derived cells had been implanted 14 days after QA-lesion (Vazey et al., 2006) raising the question of the optimal time window for transplantation. As seen by MRI imaging, shrinkage of striatal tissue occurred at around 14 days after QA-lesion. Therefore, the implantation of neural progenitor cells at this time point might influence remodelling of striatal tissue conceivably. On the other hand, TNR⁺ and the TNR⁻ cells were engrafted 3 days after lesion since such an early time point has been demonstrated to be favourable for survival and neuronal GABAergic differentiation of neural precursor cells in the QA-lesioned striatum (Bernreuther et al., 2006; Johann et al., 2007). In contrast, a study on 3-nitropropionic acid-lesioned animals showed that human neural progenitor cells
mediated neuroprotective effects on host-derived striatal neurons when cells had been implanted before the animals received a lesion (Ryu et al., 2004). These experiments show that also the time window of transplantation might affect different aspects of neuroregeneration thereby influencing functional recovery in different animal models of HD.

5.4 Outlook

This study demonstrates for the first time that genetically manipulated ESCs overexpressing a matrix molecule show beneficial effects on several aspects of stem cell-mediated tissue regeneration in a mouse model of HD. Though not mediating functional recovery, tenascin-R was shown to enhance neuronal differentiation of implanted ESC-derived cells, to enhance the recruitment of newly generated neurons in the engrafted striatum and to enhance recruitment of migrating endogenous neuroblasts from the RMS towards the lesioned striatum. This indicates that manipulating the extracellular matrix might play an important role in future therapies for human neurodegenerative diseases.

However, up to now murine and human ESCs cannot be applied for a cell replacement therapy in humans since these cells are known to cause teratoma formation after transplantation. These tumors consist of cells of the three germ layers. Teratoma formation often occurs in syngeneic transplantation paradigms whereas allogenic transplantation approaches resulted in reduced tumor formation (Björklund et al., 2002; Baier et al., 2004; Dihné et al., 2006). This observation points towards an important influence of the immune system on this severe event. Furthermore, teratoma formation is dependent on the degree of maturation of ESCs in vitro before transplantation. It was shown that engrafting stage 4 cells leads to tumor formation in about 70% of animals whereas in about 17% of animals tumors are formed when implanting ESCs differentiated for 3 days in stage 5 (Dihné et al., 2006). Prolonging the differentiation of cells in stage 5 might reduce tumor formation but is associated with decreased survival of cells. Indeed, neurons are generally regarded to be more vulnerable in comparison to immature neural precursor cells (Fricker-Gates et al., 2002; Park et al., 2007). Such high vulnerability of differentiated neuronal cells was proposed to be most likely due to expression of NMDA receptors which make them accessible to excitotoxic insults as seen in striatal lesions with the NMDA receptor agonist QA (Beal et al. 1991; Qu et al., 2003).

Interestingly, syngenic transplantation of SENAs in QA-lesioned mice was not followed by tumour formation (Dihné et al., 2006) demonstrating that changing culture conditions and
Thus maturation of cells in vitro may be a suitable approach to abolish tumorigenesis in vivo. As seen in this study, ESCs are prone to genetic manipulation providing another approach to reduce or even abolish tumor formation after transplantation. Indeed, the transfection of differentiating ESCs with reporter constructs driven by cell type-specific promoters constitutes a suitable approach to reduce heterogeneity and to remove residual tumorigenic cells due to an elimination of undifferentiated ESCs in cultures prepared for transplantation. Using sox-1-eGFP\(^+\) mouse ESCs, neuroectodermal cells expressing sox-1 and thus GFP could be purified by fluorescence-activated cell sorting (FACS). As a consequence, tumor formation was abolished when transplanted into the striatum of wildtype animals (Chung et al., 2006).

This study shows that future approaches on transplantation of ESCs should aim to improve communication between host-derived and graft-derived neurons by enhancing migration of implanted cells into the surrounding brain tissue and by promoting functional integration of these cells into basal ganglia circuitries. Furthermore, such approaches should aim at enhancing the recruitment of host-derived neuroblasts from neurogenic areas towards the non-neurogenic striatum and at mediating the integration and maturation of these cells in the basal ganglia in order to improve a stem cell-mediated neuroregeneration in HD. In this context, ectopic expression of neurotrophic factors including BDNF or matrix proteins such as reelin or tenascin-C might support regeneration in the lesioned brain, since these factors have been shown to direct detachment and migration of neural cells in vivo (Zagzag et al., 1995; Hack et al., 2002; Henry et al., 2007).
6. Summary

Huntington’s disease is a progressive neurodegenerative disorder of the central nervous system that is associated with a disturbance of motor and cognitive functions due to a degeneration of striatal GABAergic projection neurons. Since current treatments can be regarded as merely symptomatic, this study aimed to investigate the regenerative potential of murine embryonic stem cells genetically manipulated to overexpress the extracellular matrix molecule tenascin-R in a mouse model of Huntington’s disease. Different aspects of a stem cell-mediated striatal neuroregeneration were investigated that included differentiation and migration of stem cells in vitro and in vivo, generation of synaptic connections between engrafted cells and host-derived neurons, recruitment of migrating endogenous neural precursor cells, generation of host-derived newborn neurons within the striatum and functional assessment of quinolinic acid-lesioned animals treated with pre-differentiated stem cells.

In comparison to sham-transfected control cells (TNR⁻), tenascin-R overexpressing embryonic stem cells (TNR⁺) showed enhanced differentiation into neurons and a tendency towards reduced differentiation into astrocytes in vitro. Tenascin-R did not influence differentiation into neural precursor cells and their proliferation and apoptosis in vitro but inhibited migration of differentiated cells in a scratch assay in vitro. After implantation, neither TNR⁺ nor TNR⁻ stem cells exerted a positive influence on locomotor recovery of lesioned mice when compared to sham-injected control animals. However, detailed histological and stereological analysis showed that tenascin-R had positive effects on stem cell-mediated tissue regeneration in the quinolinic acid model of Huntington’s disease. One and 2 months after transplantation, tenascin-R promoted differentiation of stem cells into postmitotic neurons at the expense of astrocytes. TNR⁺ grafts contained twofold more donor-derived NeuN⁺ neurons compared to TNR⁻ grafts. Moreover, about 50% of donor-derived neurons were GABAergic in TNR⁺ and TNR⁻ grafts and thus, also the absolute number of GABAergic neurons was significantly enhanced in TNR⁺ grafts. Tenascin-R reduced the migration of implanted stem cells into the quinolinic acid-lesioned striatum and showed a tendency to enhance afferent and efferent synaptic connection between donor-derived and host-derived neurons.

Interestingly, tenascin-R influenced the recruitment of endogenous, host-derived neuroblasts after intrastrriatal implantation of stem cells. Tenascin-R promoted a stem cell-mediated attraction of migrating neuroblasts from the rostral migratory stream through the host striatum.
towards the engrafted stem cells. Furthermore, tenasin-R enhanced the number of host-derived newborn neurons within the grafted area.

These findings showed that overexpression of an extracellular matrix molecule by \textit{in vitro} pre-differentiated embryonic stem cells exerts beneficial effects on tissue regeneration in a mouse model of neurodegenerative disease.

6. \textbf{Zusammenfassung}

Der Morbus Huntington ist eine progrediente verlaufende neurodegenerative Erkrankung des zentralen Nervensystems, die aufgrund einer Degeneration striataler GABAerger Projektionsneurone mit einer Störung motorischer und kognitiver Funktionen assoziiert ist.


Im Vergleich zu Kontrollzellen (TNR⁻) zeigten Tenascin-R überexprimierende embryonale Stammzellen (TNR⁺) eine verstärkte Differenzierung in Neurone und eine Tendenz zu einer verminderten Differenzierung in Astrozyten \textit{in vitro}. Tenascin-R beeinflusste nicht die Differenzierung in neurale Vorläuferzellen sowie deren Proliferation und Apoptose \textit{in vitro}, inhibierte allerdings die Migration differenzierter Zellen in einem 'scratch assay' \textit{in vitro}.

zweimal mehr NeuN+ Neurone als Transplantate aus TNR' Zellen. Weiterhin hatten circa 50% der Neurone in TNR+ und TNR' Transplantaten einen GABAergen Phänotyp, so dass die absolute Anzahl an GABAergen Neuronen in Transplantaten aus TNR+ Zellen signifikant erhöht war. Tenascin-R inhibierte die Migration von implantierten Zellen in das mit Quinolinsäure läsionierte Striatum und zeigte eine Tendenz, die Anzahl an afferenten und efferenten Verbindungen zwischen transplantierten und empfängereigenen Neuronen zu erhöhen.


Diese Ergebnisse zeigten, dass die Überexpression eines extrazellulären Matrixproteins durch in vitro vordifferenzierte embryonale Stammzellen eine Geweberegeneration in einem Mausmodell neurodegenerativer Erkrankungen positiv beeinflussen kann.
7. Literature


Eistetter H R (1989) Pluripotent embryonal stem cell lines can be established from diaggreated mouse morulae. Dev. Growth Diff. 31:275-282


neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 118:127-38.


Appendix

1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
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<tr>
<td>A</td>
<td>adenine</td>
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<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>APS</td>
<td>ammoniumperoxodisulfate</td>
</tr>
<tr>
<td>ARPP</td>
<td>cyclic AMP-regulated phosphoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>$A_x$</td>
<td>absorbance at wavelength $x$</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor, fibroblast growth factor 2</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumine</td>
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<tr>
<td>$\beta$-tub</td>
<td>$\beta$-tubulin</td>
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<td>C</td>
<td>Cytosine</td>
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<td>CA</td>
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<tr>
<td>Camk II</td>
<td>calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
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<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<td>Cmp</td>
<td>chloramphenicol</td>
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<tr>
<td>CNPase</td>
<td>2', 3'-cyclic nucleotide 3'-phosphodiesterase</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine triphosphate</td>
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<tr>
<td>DARPP</td>
<td>dopamine and cyclic AMP-regulated phosphoprotein</td>
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<tr>
<td>dATP</td>
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<tr>
<td>dCTP</td>
<td>2'-desoxycytidinetriphosphate</td>
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<tr>
<td>dcx</td>
<td>doublecortin</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<tr>
<td>dGTP</td>
<td>2’-desoxyguanosinetriphosphate</td>
</tr>
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<td>DAPI</td>
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<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
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<td>F</td>
<td>Farad</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FNIII</td>
<td>fibronectin type III</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>g</td>
<td>g-force</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino-N-butyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GDNF</td>
<td>glia-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>hCNTF</td>
<td>human ciliary-derived neurotrophic factor</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid</td>
</tr>
<tr>
<td>HNK-1</td>
<td>human natural killer antigen-1</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LIF</td>
<td>leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LSM</td>
<td>laser scanning microscope</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>MMC</td>
<td>mitomycin C</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRT</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>neo</td>
<td>neomycin</td>
</tr>
<tr>
<td>NeuN</td>
<td>neuronal nuclei</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
</tr>
<tr>
<td>Nurr-1</td>
<td>nuclear-receptor-related-factor-1</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PGK</td>
<td>3-phospho-glycerokinase I</td>
</tr>
<tr>
<td>PLO</td>
<td>poly-L-ornithine</td>
</tr>
<tr>
<td>PNGase</td>
<td>peptide-N-glycosidase</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>QA</td>
<td>quinolinic acid</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>radioimmunoprecipitation buffer</td>
</tr>
<tr>
<td>RMS</td>
<td>rostral migratory stream</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDIA</td>
<td>stromal cell-derived inducing activity</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>stromal cell-derived factor-1α</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SENA</td>
<td>substrate-adherent embryonic stem cell-derived neural aggregate</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>STEP</td>
<td>striatal enriched phosphates</td>
</tr>
<tr>
<td>SV-2</td>
<td>synaptic vesicle protein - 2</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylendiamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosin hydroxylase</td>
</tr>
<tr>
<td>TNR</td>
<td>tenascin-R</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(-hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit (enzymatic)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>ZMNH</td>
<td>Zentrum für Molekulare Neurobiologie Hamburg</td>
</tr>
</tbody>
</table>
### 2 Oligonucleotides for sequencing

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence in 5´ - 3´- orientation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGK-end</td>
<td>GCA CGT CTG CCG CGC TGT T</td>
<td>PGK → TNR</td>
</tr>
<tr>
<td>M13-uni</td>
<td>GTA AAA CGA CGG CCA GT</td>
<td>vector → TNR</td>
</tr>
<tr>
<td>FW-1</td>
<td>GCC ACC ATG GGG ATC GAT GGG GAA ACA GT</td>
<td>TNR</td>
</tr>
<tr>
<td>FW-2</td>
<td>CTC TGA TAC TGT GGC TTT TGT GGA</td>
<td>TNR</td>
</tr>
<tr>
<td>FW-3</td>
<td>GAT AGT GGA TGC TGA GGA CAC CT</td>
<td>TNR</td>
</tr>
<tr>
<td>FW-4</td>
<td>TTA CCT TCA CCC ACA AGA TCA ACC T</td>
<td>TNR</td>
</tr>
<tr>
<td>FW-5</td>
<td>AGT GTC AAT ATC ACC TGG AGT GAC</td>
<td>TNR</td>
</tr>
<tr>
<td>FW-6</td>
<td>GTG AAC GCA TCT GCA CCC TGG T</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-1</td>
<td>GAA TTT CAA GGC TCG CCG TTT CC</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-2</td>
<td>TGT TCT CTG ACA GGC CCT CTA GT</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-3</td>
<td>CCT CCG CCA CCC ACC AAG CCA</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-4</td>
<td>GTC TCT GTG ATC GTC TTG AAC TGT</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-5</td>
<td>ATC AAG ACA GCG TGC CTC TTG GT</td>
<td>TNR</td>
</tr>
<tr>
<td>RV-6</td>
<td>GAT CTT GTA CAG GCT TCT GCT GT</td>
<td>TNR</td>
</tr>
</tbody>
</table>

**Table 5:** Oligonucleotides used for sequencing of the p901-PGK-neo-PGK-TNRmyc construct
3 Maps of plasmids

Figure 32: Maps of plasmids used for transfection of embryonic stem cells.
Acknowledgements

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Curriculum vitae

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First name:              Gunnar
Day of birth:              27.09.1976
Place of birth:          Eutin (Germany)

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since July 2004

PhD thesis
PhD-student at the Institute for the Biosynthesis of Neural Structures (chair: Prof. Dr. M. Schachner) of the Center for Molecular Neurobiology Hamburg (Germany):
Topic: Analysis of murine embryonic stem cells overexpressing the extracellular matrix molecule tenascin-R \textit{in vitro} and after transplantation in a mouse model of Huntington’s disease

03/2001 – 07/2004

Medical doctoral thesis
Doctoral student at the Institute for Medical Molecular Biology, Medical University of Lübeck (chair: Prof. Dr. P.K. Müller);
research group \textit{Cellular Differentiation} (Prof. Dr. J. Rohwedel):
Topic: The function of the transcription factor Sox9 during chondrogenic differentiation – analysis of Sox9 deficient murine embryonic stem cells \textit{in vitro}.
Defence of this thesis in April 2005: \textit{summa cum laude}

Education


PhD-program \textit{Systems Neuroscience} at the Center for Systems Neuroscience Hannover (Germany)


Graduate program \textit{Molecular Biology} at the Center for Molecular Neurobiology Hamburg, University of Hamburg (Germany)

10/1997 – 06/2004

Medical education at the Medical University of Lübeck (Germany)
Examinations: preliminary medical examination (physikum): 09/1999
1.state examination: 08/2000
2.state examination: 03/2003
3.state examination: 06/2004
Final grade: 2
Curriculum vitae

Final year: Internal Medicine: General Hospital, Lübeck; Prof. Hütteroth (04/2003-03/2004; 4 months each)
Surgery: Sligo General Hospital, Sligo (Ireland); Mr. Martin Caldwell (Consultant Surgeon)
Neurology: Medical University of Lübeck; Prof. Kömpf

Clinical rotations:
- 09/2002 Internal medicine, General Hospital, Lübeck
- 08/2002 ENT, Medical University of Lübeck
- 03/2002 Anaesthesics, Bedford General Hospital, Bedford (England)
- 02/2002 Neurosurgery, Medical University of Lübeck
- 09/2000 Surgery, Nordstadt-Hospital, Hannover (Germany)

Research courses and seminars:
- 10/2002 Institute for Biochemistry/Neurological Sciences, University of Verona (Italy)
- 1998/1999 Immunochemical seminar and
- 02/03 1999 Immunochemical course, Department of Immunochemistry and Biochemical Microbiology, Research Center Borstel (Germany)
- 09/10 1998 Institute for Immunology and Transfusion Medicine, Medical University of Lübeck

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Graduation, grade: 1,8
1996
1983-1987
Primary school in Eutin

Civil service
- 1996-1997 nursing in St. Elisabeth-Hospital Eutin
List of own publications

Original articles:


6. Jakovcevski, I.*, Siering*, J., Hargus*, G., Hoelters, L., Yin, S., Schachner, M., Irintchev, A.: Close homologue of L1 regulates numbers of Purkinje and granule cells during cerebellum development in mice (submitted for publication); *equal contribution


Reviews:


Presentation of this research project

1. NECTAR conference in Freiburg (Germany), November 30th to December 2nd 2006

2. Retreat of the Center for Molecular Neurobiology Hamburg (ZMNH) in Jesteburg (Germany), May 14th to May 15th 2007

3. Center for Neuroregeneration Research, McLean Hospital, Harvard Medical School, Belmont (USA), June 8th 2007

4. Life and Brain Center Bonn, University of Bonn (Germany), September 6th 2007