Cell-based therapy in epilepsy:

Anticonvulsant efficacy and risk assessment of grafting genetically engineered inhibitory cell lines and neuronal precursor cells into the rat subthalamic nucleus
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Für meine Eltern
SUMMARY ............................................................................................................................... 1

ZUSAMMENFASSUNG ............................................................................................................. 3

1 GENERAL INTRODUCTION ................................................................................................. 5

1.1 Epilepsy ............................................................................................................................. 7
  1.1.1 Definition and Significance .......................................................................................... 7
  1.1.2 Pharmacoresistant Epilepsies and Alternative Treatment Strategies ....................... 8

1.2 Neurotransplantation in Epilepsy Research ..................................................................... 9

1.3 Role of Basal Ganglia in Seizure Modulation ................................................................. 10

1.4 Neurotransplantation into Basal Ganglia in Epilepsy Research ..................................... 13
  1.4.1 Transplantation of Neuronal Precursor Cells into Basal Ganglia in Epilepsy Research ......................................................... 14
  1.4.2 Transplantation of GABAergic Cell Lines into the Basal Ganglia in Epilepsy Research ......................................................................................................................... 15
  1.4.3 The Subthalamic Nucleus as Target Region for Neurotransplantation ............... 16

1.5 Immunomodulation to Prevent Graft Rejection ......................................................... 18
  1.5.1 Pharmacological Immunosuppression ....................................................................... 19
  1.5.2 Induction of Immunological Tolerance .................................................................... 21

1.6 Timed Intravenous Pentylenetetrazole Seizure Threshold Test and
Amygdala-Kindling Model ................................................................................................. 22

1.7 Aim of the Studies ............................................................................................................ 23
  1.7.1 Study 1: Grafting GABAergic Cells into the Subthalamic Nucleus .................... 23
  1.7.2 Study 2: Effects of Cyclosporine A on Seizure Thresholds .................................... 24
  1.7.3 Study 3: Porcine Cell Grafting and Induction of Tolerance ................................. 24
# Table of Contents

2 Anticonvulsant Effects by Bilateral and Unilateral Transplantation of GABA-Producing Cells into the Subthalamic Nucleus in an Acute Seizure Model

3 Comprehensive Study of Acute and Chronic Treatment with Different Preparations, Doses, and Administration Routes of Cyclosporine A on Seizure Thresholds and Adverse Effects

## 3.1 Abstract

## 3.2 Introduction

## 3.3 Experimental Procedures

### 3.3.1 Animals

### 3.3.2 Timed Intravenous PTZ Seizure Threshold Test

### 3.3.3 Implantation of Kindling Electrode and Kindling

### 3.3.4 CsA Treatment Regimens

### 3.3.5 CsA Treatment in the PTZ Model

### 3.3.6 CsA Treatment in the Kindling Model

### 3.3.7 Blood Sampling and Whole Blood Drug Analysis

### 3.3.8 Behavioral Testing Battery and Physiological Measures

### 3.3.9 Histological Verification of Kindling Site

### 3.3.10 Statistics

## 3.4 Results

### 3.4.1 CsA Whole Blood Concentration

### 3.4.2 Basal (pre-drug) PTZ Seizure Thresholds

### 3.4.3 Lack of Robust Acute and Chronic Effects of CsA on PTZ Seizure Thresholds

### 3.4.4 Kindling Development and Basal (pre-drug) Kindling Parameters

### 3.4.5 Lack of Acute and Chronic Effects of CsA on Kindled Seizure Parameters
3.4.6 Effects of CsA on Rat Behavior.............................................. 47
3.4.7 Effects of CsA on Defecation................................................. 50
3.4.8 Effects of CsA on Body Temperature................................... 50
3.4.9 Effects of CsA on Body Weight .......................................... 51

3.5 Discussion .................................................................................. 52
3.5.1 Lack of Robust CsA Effects on Seizure Thresholds............... 52
3.5.2 Bioavailability of CsA............................................................ 55
3.5.3 Adverse Effects Induced by CsA........................................... 55

3.6 Conclusion .................................................................................. 56

3.7 References .................................................................................. 58

4 IMMUNOMODULATION FOR LONG-LASTING ANTICONVULSANT EFFECTS AFTER NEURAL XENOGRAFTING IN RATS – NEONATAL INDUCTION OF TOLERANCE VERSUS PHARMACOLOGICAL IMMUNOSUPPRESSION .................................................. 63

4.1 Abstract ..................................................................................... 64

4.2 Introduction ................................................................................ 64

4.3 Material and Methods ................................................................. 66
4.3.1 Animals................................................................................ 67
4.3.2 Cell Cultivation...................................................................... 67
4.3.2.1 Preparation of Primary Cell Culture .................................. 68
4.3.2.2 Preparation of pNPCs for Tolerance Induction and
Neurotransplantation .................................................................. 68
4.3.3 Induction of Immunological Tolerance .................................. 69
4.3.4 Pharmacological Immunosuppression ................................... 69
4.3.5 PTZ Seizure Threshold Test .................................................. 69
4.3.6 Transplantation of Porcine Neuronal Precursor Cells .......... 71
4.3.7 Behavioral Tests ................................................................. 71
4.3.7.1 Open Field .................................................................... 72
4.3.7.2 *Elevated Plus Maze Test* ................................................................. 73
4.3.8 Histological Verification of Graft Localization ........................................... 74
4.3.9 Statistical Analysis ..................................................................................... 74

**4.4 Results** .......................................................................................................... 75

4.4.1 Localization of Grafted pNPCs ................................................................. 75
4.4.2 Basal (control) PTZ Seizure Thresholds ..................................................... 75
4.4.3 Anticonvulsant Effects of pNPC Transplantation in the PTZ Seizure Threshold Test ........................................................................................................ 76
4.4.4 Behavioral Tests ......................................................................................... 77
4.4.4.1 *Body Weight and Body Temperature* .................................................. 77
4.4.4.2 *Behavior in the Open Field* ................................................................ 78
4.4.4.3 *Behavior in the Elevated Plus Maze* .................................................. 78

**4.5 Discussion** ..................................................................................................... 80

4.5.1 Anticonvulsant Efficacy of pNPCs Grafted into the STN ............................. 80
4.5.2 Adverse Effects caused by CsA treatment ................................................... 83

**4.6 Conclusion** ..................................................................................................... 84

**4.7 References** .................................................................................................... 85

**5 General Discussion** .......................................................................................... 89

5.1 Anticonvulsant Efficacy of GABAergic Cell Lines and Porcine Neuronal Precursor Cells Transplanted into Rats .................................................................... 89
5.1.1 Anticonvulsant Effects of a Bilateral Neurotransplantation into the Subthalamic Nucleus ............................................................................................. 89
5.1.2 Anticonvulsant Effects of an Unilateral Transplantation into the Subthalamic Nucleus ............................................................................................. 90
5.1.3 Lack of Long-lasting Anticonvulsant Effects ............................................. 92

5.2 Necessity, Efficacy, and Safety of an Immunomodulation for Neurotransplantations in Experimental Epilepsy Research .............................................. 93
5.2.1 Tissue Reactions after Neurotransplantation of hGAD-overexpressing Cells ........................................................................................................................................................................... 93
5.2.2 Cyclosporine A does not Robustly Influence Seizure Thresholds .......... 94
5.2.3 Adverse Effects of Treatment with Cyclosporine A ................................. 95
5.2.4 Comparison of Immunomodulatory Strategies to Promote and Prolong the Anticonvulsant Efficacy of Grafted Porcine Neuronal Precursor Cells ........................................................................................................................................................................... 97

5.3 Localization of the Neural Grafts – Methodical Issues ................................. 98

5.4 Conclusions and Outlook ............................................................................. 100

6 REFERENCES ........................................................................................................... 101

7 SUPPLEMENTS ........................................................................................................ 119

ACKNOWLEDGEMENTS ............................................................................................... 121
# Abbreviation List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADT</td>
<td>afterdischarge threshold</td>
</tr>
<tr>
<td>ADD</td>
<td>afterdischarge duration</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
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<tr>
<td>EPM</td>
<td>elevated plus maze</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GST</td>
<td>generalized seizure threshold</td>
</tr>
<tr>
<td>hGAD</td>
<td>human glutamic acid decarboxylase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LGE</td>
<td>lateral ganglionic eminence</td>
</tr>
<tr>
<td>LP</td>
<td>latency period</td>
</tr>
<tr>
<td>MGE</td>
<td>medial ganglionic eminence</td>
</tr>
<tr>
<td>OF</td>
<td>open field</td>
</tr>
<tr>
<td>pCsA</td>
<td>pure substance CsA</td>
</tr>
<tr>
<td>pNPC</td>
<td>porcine neuronal precursor cell</td>
</tr>
<tr>
<td>post</td>
<td>post-treatment</td>
</tr>
<tr>
<td>PTZ</td>
<td>pentylenetetrazole</td>
</tr>
<tr>
<td>Sand</td>
<td>Sandimmune® (Novartis Pharmaceuticals)</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>seizure duration</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra pars reticulata</td>
</tr>
<tr>
<td>SS</td>
<td>seizure severity</td>
</tr>
<tr>
<td>STN</td>
<td>subthalic nucleus</td>
</tr>
<tr>
<td>Tol</td>
<td>neonatally induced tolerance</td>
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Annelie Handreck

Cell-based therapy in epilepsy: Anticonvulsant efficacy and risk assessment of grafting genetically engineered inhibitory cell lines and neuronal precursor cells into the rat subthalamic nucleus

About 30% of patients suffering from epilepsy are considered pharmacoresistant, meaning that seizures occur despite appropriate treatment with antiepileptic drugs. Neural transplantation of inhibitory cells into brain regions involved in seizure generation or propagation is one promising experimental approach to overcome this problem. Apart from directly targeting the seizure focus, grafting appropriate cells into remote structures known to be crucially involved in seizure modulation may be an advantageous strategy, especially for pharmacoresistant patients with multiple epileptic foci or without clear focal onset, i.e. for patients in which focus resection is not feasible. Clinical experience with deep brain stimulation together with a recent microinjection study in rats indicated that the subthalamic nucleus (STN), a key basal ganglia structure, might be a highly promising target in this respect. However, until now the STN has not been targeted for neurotransplantations in experimental epilepsy research.

In a first proof-of-principle study, we therefore grafted γ-aminobutyric acid (GABA)-producing striatal rat cell lines into the STN of rats. Anticonvulsant efficacy was evaluated using an acute seizure model, the intravenous pentylenetetrazole (PTZ) seizure threshold test. Bilateral and unilateral transplantation of GABAergic cell lines into the STN caused clear anticonvulsant effects. Grafting the GABAergic cells bilaterally outside the STN and intrasubthalamic grafting of control cells, which do not produce GABA, did not induce anticonvulsant effects, emphasizing the site- and cell-specificity of the observed anticonvulsant effects. In line with previous transplantation studies targeting other basal ganglia regions in experimental epilepsy and despite graft survival up to five weeks after transplantation, the anticonvulsant effects were only transient.

For clinical translation, other cell sources such as porcine neuronal cells have to be investigated preclinically to demonstrate safety and efficacy after grafting into the brain.
xenotransplantation studies, an immunosuppression is necessary to prevent graft rejections. However, conflicting data from literature indicated that a treatment with the commonly used immunosuppressive drug cyclosporine A (CsA) might itself act pro- or anticonvulsant in different experimental seizure and epilepsy models. In a second study, we therefore comprehensively investigated the effects of an acute and chronic immunosuppression with different doses, application routes, and preparations of CsA on seizure thresholds in the PTZ seizure threshold test in rats. We included investigations of CsA effects using the amygdala-kindling model as a chronic epilepsy model. Independent of CsA treatment regimen, no robust effects on seizure thresholds were observed, indicating that an immunosuppression with CsA might be a safe and feasible option for use in neural transplantation experiments in the two models. However, observed adverse effects included transient gastrointestinal problems, a decrease in body temperature, and reduced locomotion.

An interesting alternative immunomodulatory approach is the neonatal induction of tolerance towards the cells intended for later transplantation. In a third study, porcine neuronal precursor cells (pNPCs) were therefore comparatively grafted into the STN of rats, which received either a pharmacological immunosuppression with CsA, were neonatally tolerance-induced, or were not immunomodulated at all. Bilateral grafting of pNPCs into the STN was anticonvulsant in all animal groups, but only the induction of tolerance was able to promote long-lasting anticonvulsant effects in response to grafting of pNPCs. Compared to CsA treatment, the induction of tolerance did not cause any obvious adverse effects. Nevertheless, the observed anticonvulsant effects were still transient. Our promising data on neonatal induction of tolerance and the use of pNPCs for xenotransplantation studies in epilepsy research merit further investigations.
**ZUSAMMENFASSUNG**

Annelie Handreck

**Zellbasierte Therapie bei Epilepsie: Antikonvulsive Wirksamkeit und Risikoabschätzung der Transplantation gentechnologisch modifizierter, inhibitorischer Zelllinien und neuronaler Vorläuferzellen in den subthalamischen Nukleus von Ratten**


andere Basalganglienregionen, waren die antikonvulsiven Effekte nur transient, obwohl die Zellen bis fünf Wochen nach der Transplantation überlebten.


1 GENERAL INTRODUCTION

Epilepsies are among the most common neurological disorders of the central nervous system and occur with a prevalence of 1-2% (Löscher and Schmidt, 2002). They are characterized by repeated spontaneous seizures of central origin caused by an overexcitability of neurons due to an imbalance between excitatory and inhibitory neurotransmission in several brain regions (Scharfman, 2007). Additionally, epilepsies are often accompanied by psychiatric comorbidities like depression and behavior modifications (Kanner, 2009). About 30% of patients suffering from epilepsy are considered pharmacoresistant, meaning that seizures occur despite appropriate treatment with antiepileptic drugs. Furthermore, currently available antiepileptic drugs reduce the symptoms instead of providing a cure (Schmidt and Löscher, 2005; Shorvon, 2009; Löscher and Schmidt, 2011). Therefore, the investigation and development of alternative treatment strategies for pharmacoresistant patients is a major challenge in epilepsy research.

Among others, neural transplantation into the basal ganglia, known to be involved in remote modulation of seizures emanating from the limbic system, is a promising experimental approach to treat pharmacoresistant epilepsies (Löscher et al., 2008; Al-Otaibi et al., 2011). The aim is to permanently suppress epileptic seizures by grafting appropriate cells, which express the inhibitory neurotransmitter γ-aminobutyric acid (GABA), into specific brain regions to restore the balance between inhibitory and excitatory neurotransmission (Löscher et al., 2008; Nolte et al., 2008; Thompson, 2009).

The basal ganglia are involved in propagation and modulation of seizures emanating from the limbic system, rendering them promising targets for focal seizure manipulation (Gale et al., 2008; Löscher et al., 2008). Previous studies showed that transplantation of fetal GABAergic precursor cells (Löscher et al., 1998) and genetically engineered GABA-producing cells (Thompson et al., 2000; Thompson and Suchomelova, 2004; Castillo et al., 2008; Nolte et al., 2008; Gernert et al., 2011) into the substantia nigra pars reticulata (SNr), a basal ganglia output structure, caused anticonvulsant, albeit transient, effects. A recent pharmacological study of our group indicated that the subthalamic nucleus (STN), which regulates the activity of the SNr by providing excitatory glutamatergic input, might be an even more promising target region in this respect than the SNr (Bröer et al., 2012). Because
functional neurosurgery of the STN is already clinically established (Benabid, 2007; Al-Otaibi et al., 2011), a clinical translation of grafting approaches targeting the STN seems realistic. Nevertheless, until now no studies investigated the efficacy of grafting GABAergic cells into the STN in epilepsy models.

In a first proof-of-principle study, we therefore grafted different GABAergic cells and a non-GABAergic control cell line bilaterally into the STN of rats. The anticonvulsant efficacy was assessed using an acute seizure model. Depending on the grafted cell type (e.g. xenotransplantation), an immunosuppression is necessary to prevent graft rejection (Larsson and Widner, 2000; Wennberg et al., 2001; Kahan, 2009) and to promote long-term anticonvulsant effects. However, conflicting data indicate that a treatment with the commonly used immunosuppressive drug cyclosporine A (CsA) might itself act pro- or anticonvulsant in different rat seizure and epilepsy models (Racusen et al., 1988; Moia et al., 1994; Asanuma et al., 1995b; Setkowicz and Ciarach, 2007).

In a second study we therefore comprehensively investigated putative influences of an immunosuppressive treatment with different doses, application routes, and preparations of CsA on seizure thresholds in an acute seizure model and in a chronic epilepsy model in rats. Acute and chronic CsA treatment regimens were considered. Additionally, behavioral tests and physiological measures were conducted to detect putative adverse effects of CsA treatment. Finally, whole blood CsA levels were analyzed.

In a final step, we investigated the influence of different immunomodulatory strategies on graft-induced modulations of seizure thresholds in an acute seizure model and conducted behavioral test to detect putative adverse effects. Therefore, we grafted porcine neuronal precursor cells (pNPCs) into the STN of rats. To compare different immunomodulatory strategies for preventing graft rejection, the cells were transplanted into rats that either received a conventional pharmacological immunosuppression with CsA or in which tolerance was induced neonatally (Kelly et al., 2009).

The aim of our studies was to investigate the anticonvulsant efficacy and related risks of neural transplantation of genetically engineered GABAergic cell lines and pNPCs into the STN using experimental seizure and epilepsy models. For safe xenotransplantations in epilepsy research, we further aimed to find an immunosuppression protocol that does not influence seizure susceptibility in rats and shows minimum adverse effects. Additionally, we wanted to
compare a pharmacological immunosuppression with an induction of immunological tolerance with regard to the potential of promoting graft survival and long-lasting anticonvulsant effects.

1.1 Epilepsy

1.1.1 Definition and Significance

The word epilepsy originates from the Greek and means "to seize, possess, or afflict" (Magiorkinis et al., 2010). According to the International League against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE), epilepsy is defined as a brain disorder that is characterized by a permanent predisposition for the development of epileptic seizures, often accompanied by neurobiological, cognitive, and social consequences, as well as psychiatric comorbidities like depression, psychoses, and behavioral modifications (Fisher et al., 2005; Kanner, 2009). Epilepsies are characterized by repeated spontaneous seizures with central origin caused by an overexcitability and synchronized activity of neuron populations. This leads to an imbalance between excitatory and inhibitory neurotransmission in several brain regions (Scharfman, 2007).

Epilepsies are among the most common neurological disorders of the central nervous system (CNS) and emerge with a prevalence of 1-2% in humans and in animals, especially in dogs and cats (Löscher and Schmidt, 2002; Chandler, 2006). Affected patients not only suffer from a reduced quality of life, epilepsies can also cause substantial damage of the brain and, if untreated, can lead to death (Löscher and Schmidt, 2002; Engel et al., 2003).

The causes of epilepsies are classified as genetic, structural-metabolic, and unknown (Berg et al., 2010). Structural-metabolic epilepsies emerge from a disease or injury of the CNS, meaning an initial insult such as infection, birth incident, neurodegeneration, status epilepticus, brain trauma, stroke, or brain tumor. Furthermore, several types of seizures can be differentiated. Focal (partial) seizures emanate from a discrete brain region in one hemisphere and are characterized by spontaneous motor, sensory, and/or vegetative symptoms. Consciousness can be impaired or unimpaired. Focal seizures often show a secondary generalization, meaning that the epileptic activity propagates from the focus to both hemispheres. In contrast, primary generalized seizures always have their origin in both...
hemispheres. In primary generalized seizures, myoclonic, clonic, tonic-clonic seizures, and absences can occur (Fisher et al., 2005; Engel, 2006; Berg et al., 2010).

Temporal lobe epilepsy is the most common and difficult to treat type of epilepsy and is characterized by partial seizures, often with secondary generalization. This type of epilepsy is associated with neurodegeneration, erroneous release and function of neurotransmitters, and structural and cellular changes in the hippocampus and other regions (Scharfman, 2007). The amygdala-kindling model and different post-status-epilepticus models are commonly used as animal model for temporal lobe epilepsy (Löscher, 2002).

1.1.2 Pharmacoresistant Epilepsies and Alternative Treatment Strategies

Currently available antiepileptic drugs concentrate on reducing the symptoms by suppressing seizures, but they do not provide a cure for epilepsies. Furthermore, the systemic administration of antiepileptic drugs often induces substantial adverse effects (Löscher and Schmidt, 2002). Finally, despite adequate treatment with antiepileptic drugs, about 30% of patients suffer from intractable, pharmacoresistant epilepsy. In temporal lobe epilepsy, the amount of pharmacoresistant patients is about 60-70% and thus even twice as high (Schmidt and Löscher, 2005; French, 2007; Brodie, 2010; Alvarez-Dolado and Broccoli, 2011; Löscher and Schmidt, 2011). Pharmacoresistance means that, despite appropriate treatment with two different antiepileptic drugs (in monotherapy or combination therapy), patients do not become seizure free (Kwan et al., 2010). To achieve seizure freedom in those patients, in some cases a surgical resection of the epileptic focus is feasible. Although the patients still have to be treated to control the seizures, they now respond to antiepileptic drugs (Wiebe et al., 2001). This indicates that specific brain areas are still organized in an epileptic network despite the resection of the focus. Moreover, a focus resection is only appropriate if the focus can be identified unambiguously and is not part of functional brain tissue (Löscher and Schmidt, 2002; Duncan, 2011). Among other treatment strategies (e.g. vagus nerve stimulation, deep brain stimulation, transcranial magnetic stimulation, and ketogenic diet), the transplantation of appropriate neuronal cells into brain regions involved in seizure modulation is a promising experimental approach to treat pharmacoresistant epilepsies (Schachter et al., 1998; Raedt et al., 2007; Löscher et al., 2008; Boon et al., 2009; Nitsche and Paulus, 2009; Al-Otaibi et al., 2011; Sebe and Baraban, 2011).
1.2 Neurotransplantation in Epilepsy Research

For neurotransplantation in epilepsy research, cells are grafted into specific brain regions with the aim to induce a long-lasting seizure suppression. In contrast to systemic applications of antiepileptic drugs, localized neurotransplantation can be restricted to brain regions involved in seizure modulation, thereby causing fewer adverse effects. The idea is that grafted cells either functionally replace and repair damaged host brain tissue or restore the balance between inhibitory and excitatory neurotransmission by producing specific transmitters (Lösch et al., 1998; Gernert et al., 2002; Turner and Shetty, 2003; Raedt et al., 2007; Shetty and Hattiangady, 2007; Castillo et al., 2008; Nolte et al., 2008; Thompson, 2009; Waldau et al., 2010; Sebe and Baraban, 2011; Anderson and Baraban, 2012).

Beginning in the 1980s, the first experimental neural transplantation studies were conducted in neurodegenerative diseases with selective loss of neurons such as in Parkinson’s disease. Studies in animal models of Parkinson’s disease revealed promising results (Brundin et al., 1985a; Brundin et al., 1985b; Brundin et al., 1986; Wictorin et al., 1992; Olsson et al., 1997; Björklund and Lindvall, 2000; Armstrong et al., 2003) and thus led to numerous clinical studies, albeit with inconsistent success (Kordower et al., 1995; Björklund and Lindvall, 2000; Schumacher et al., 2000; Dunnett et al., 2001; During et al., 2001; Freeman et al., 2001; Anderson and Caldwell, 2007; Olanow et al., 2009; Björklund and Kordower, 2013), emphasizing the as yet rather experimental status of this treatment strategy.

In experimental epilepsy research, one obvious strategy for neural transplantation approaches is to graft appropriate cells into the seizure focus (Turner and Shetty, 2003; Raedt et al., 2007; Lösch et al., 2008; Hunt et al., 2013). Limbic regions, such as the hippocampus of patients suffering from temporal lobe epilepsy, undergo massive structural changes including neurodegeneration. Consequently, in the few epilepsy patients which have been grafted with porcine inhibitory precursor cells as a first proof-of-principle clinical trial, the seizure focus was targeted as an attempt to achieve brain repair (Schachter et al., 1998).

In experimental epilepsy models, grafting of inhibitory cells into the seizure focus (Turner and Shetty, 2003; Raedt et al., 2007; Hunt et al., 2013) or into regions close to the focus (Gernert et al., 2002) have been intensely investigated. Especially precursor cells of the medial or lateral ganglionic eminence (MGE and LGE, respectively) have been repeatedly
used for transplantation. These regions belong to the ventral subpalladium of the telencephalon and migrate during the brain development into the cortex, hippocampus, basal ganglia regions, and bulbus olfactorius, where they form GABAergic projection neurons and interneurons (de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Anderson et al., 2001; Mandal et al., 2013). Cells of the MGE are the main source of cortical GABAergic interneurons and show a high potential for migration even after grafting into the adult brain. This may enable the cells to integrate into damaged brain regions and restore their function (Wichterle et al., 2001; Anderson and Baraban, 2012). In different studies, MGE precursor cells were transplanted into the hippocampus of mice and rats and yield a high percentage of differentiation into GABAergic cells. These cells showed functional integration and caused long-lasting anticonvulsant effects (Wichterle et al., 2001; Baraban et al., 2009; Zipancic et al., 2010; Hattiangady and Shetty, 2011; Anderson and Baraban, 2012; Hunt et al., 2013).

However, especially for patients with multiple epileptic foci or with unclear focal onset of seizures, other strategies have to be investigated. Here, grafting appropriate cells into remote brain structures involved in seizure propagation and modulation is an advantageous strategy. In this respect, basal ganglia regions are highly promising, because they are involved in seizure propagation, generalization, and modulation (DeLong and Wichmann, 2007; Gale et al., 2008; Löscher et al., 2008), and show plastic network changes in response to repeated seizures (Gernert et al., 2004; Kücker et al., 2010; Töllner et al., 2011).

1.3 Role of Basal Ganglia in Seizure Modulation

Seizures emanating from the limbic system propagate and generalize via specific anatomic paths, which also involve the basal ganglia. They are a group of subcortical nuclei that physiologically convey mainly motor, but also cognitive and limbic functions (Bolam et al., 2000; DeLong and Wichmann, 2007; Al-Otaibi et al., 2011). Basal ganglia regions are part of the epileptic network and thus have been shown to be subject to plastic network changes upon repeated seizure activity (Gernert et al., 2004; Nolte et al., 2006; Kücker et al., 2010). Furthermore, they have been reported to be involved in mechanisms of pharmacoresistance (Töllner et al., 2011). The basal ganglia include the substantia nigra (consisting of pars compacta and pars reticulata), the striatum (consisting of the nucleus caudatus and
putamen), the globus pallidus (rat; globus pallidus externus in humans), the entopeduncular nucleus (rat; globus pallidus internus in humans), and the STN. The basal ganglia regions are anatomically and functionally connected with each other and with the limbic system (Fig. 1). Dysfunctions of the basal ganglia can lead to neuropsychiatric symptoms, cognitive changes, and hypo- or hyperkinetic movement disorders. The most common symptoms can be seen in Morbus Parkinson (tremor, bradykinesia, rigor, and bent body posture) and Chorea Huntington (extensive, involuntary movements) (DeLong, 2000). Physiologically, the basal ganglia have a gating function, meaning that they channel wanted movements and inhibit involuntary activities.

The basal ganglia network consists of parallel loops, which go from the cortex over the basal ganglia to the brain stem and the thalamus and from there back to the cortex. In temporal lobe epilepsies, seizures emanate from a focus within the temporal lobe (usually the hippocampus, the entorhinal cortex, or the amygdala). During secondary generalization (Fig. 1), seizure activity is transmitted from the focus via different routes to the basal ganglia, for example over the cortex to the striatum, which is considered as the entry gate of the basal ganglia network. The seizure propagation continues from the striatum to the SNr via a direct or indirect path. The direct striato-nigral projection is monosynaptic and utilizes the inhibitory neurotransmitter GABA. The activity of GABAergic striatal projection neurons (medium-sized spiny neurons) therefore results in an increased concentration of GABA in the SNr, which leads to an inhibition of nigral neurons (Hattori et al., 1973; Fonnum et al., 1978; Bolam et al., 2000; Gale et al., 2008).

In contrast, the indirect striato-nigral path consists of three interconnections. The striatum innervates the globus pallidus (externus) via GABAergic projection neurons (medium-sized spiny neurons). From there, activity is transmitted again via GABAergic projections to the STN. The projection neurons of the STN innervate the SNr and lead to an increased activity of nigral neurons due to the release of the excitatory neurotransmitter glutamate. The STN is additionally regulated by further efferences such as from the cortex and the pedunculopontine nucleus (Alexander and Crutcher, 1990; Robledo and Feger, 1990; Bolam et al., 2000; Shen and Johnson, 2006; DeLong and Wichmann, 2007; Deniau et al., 2007; Gale et al., 2008).
Together with the entopeduncular nucleus, the SNr serves as an output gate of the basal ganglia. The SNr consists to 90% of GABAergic neurons and forwards information to different regions including thalamus, rostral colliculus (colliculus superior in humans), and pedunculopontine nucleus (Fonnum et al., 1978; Alexander and Crutcher, 1990; Bolam et al., 2000; Gale et al., 2008). The SNr is one of the best-studied basal ganglia structures with regard to its function in propagation and modulation of seizures. Moreover, it is a well-investigated basal ganglia target for experimental, therapeutic manipulations in epilepsy research (Iadarola and Gale, 1982; Garcia-Cairasco and Sabbatini, 1983; Le Gal La Salle et al., 1983; McNamara et al., 1984; De Sarro et al., 1991; Moshé et al., 1992; Depaulis et al., 1994; Gernert and Löscher, 2001; Gale et al., 2008; Löscher et al., 2008; Nolte et al., 2008; Thompson, 2009; Bröer et al., 2012). Due to reciprocal connections of basal ganglia and thalamus with the limbic system (Fig. 1), a direct modulation of seizure initiation in the limbic system can be achieved (Löscher et al., 2008).

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**Fig. 1:** Schematic illustration of focal (limbic) and secondary generalized seizure propagation pathways in temporal lobe epilepsy. Emanating from the focus, which is located within the limbic system (light pink), seizures propagate via the cortex (blue), the basal ganglia (yellow), and their downstream structures (green). Several transmitters including γ-aminobutyric acid (GABA; red arrows) and glutamate (blue arrows) are involved in that epileptic network. Modified from Löscher et al., 2008.
It is assumed that an increased inhibition of the SNr leads to a disinhibition of downstream structures and thereby prevents the generalization of epileptic seizures and, via the reciprocal connections with the limbic system, raises the threshold for seizure induction. This can be achieved by a direct inhibition of the SNr or indirectly via reduction of excitatory input from the STN. This principle of seizure reduction due to an increased direct inhibition of the SNr (Iadarola and Gale, 1982; McNamara et al., 1984; Dybdal and Gale, 2000; Bröer et al., 2012) or an indirect inhibition of the SNr by inhibiting the STN (Deransart et al., 1996; Velísková et al., 1996; Deransart et al., 1998; Dybdal and Gale, 2000; Bröer et al., 2012) has been repeatedly used in local pharmacological or electrical modulations of the epileptic network. Even though this approach was also used for transplantation of inhibitory cells into the SNr (Löscher et al., 1998; Castillo et al., 2008; Löscher et al., 2008; Nolte et al., 2008; Thompson et al., 2009), the STN has not been targeted for neurotransplantation studies so far (Fig. 3, Page 17).

1.4 Neurotransplantation into Basal Ganglia in Epilepsy Research

Although the basal ganglia have been repeatedly shown to be subject to seizure-induced network changes (Gernert et al., 2004; Kücker et al., 2010; Töllner et al., 2011), neurodegenerations are rather limited and strongly depend on the used animal model (Freichel et al., 2004). The transplantation into rather intact brain regions is controversially discussed. Cells grafted into the epileptic hippocampus shortly after the insult showed a higher survival rate than cells transplanted into the hippocampus of healthy animals (Zaman et al., 2001; Zaman and Shetty, 2002). However, as mentioned earlier, the basal ganglia are part of the epileptic network and are thus involved in the propagation and generalization of seizures, epilepsy-induced plastic network changes, and due to reciprocal connections to the limbic system are also involved in the modulation of seizures emanating from the limbic system (Iadarola and Gale, 1982; Gernert et al., 2004; Löscher et al., 2008; Cremer et al., 2009). Additionally, neural transplantation into the basal ganglia has an unrivaled advantage. Animal experiments proved that different types of seizures or epilepsies with various localizations of the focus can be suppressed by using the basal ganglia as target region for pharmacological manipulations (Iadarola and Gale, 1982; Garcia-Cairasco and Sabbatini, 1983; Le Gal La Salle et al., 1983; Albala et al., 1984; De Sarro et al., 1984; McNamara et al.,
In the previously conducted neurotransplantation studies, different cell types as well as different epilepsy and seizure models were used. Cell types grafted in these studies were primarily fetal cells (Lösch et al., 1998) or genetically engineered cells (Thompson et al., 2000; Castillo et al., 2008; Nolte et al., 2008; Thompson, 2009; Gernert et al., 2011). In the previously conducted neurotransplantation studies, different cell types as well as different epilepsy and seizure models were used. Cell types grafted in these studies were primarily fetal cells (Lösch et al., 1998) or genetically engineered cells (Thompson et al., 2000; Castillo et al., 2008; Nolte et al., 2008; Thompson, 2009; Gernert et al., 2011). In the previously conducted neurotransplantation studies, different cell types as well as different epilepsy and seizure models were used. Cell types grafted in these studies were primarily fetal cells (Lösch et al., 1998) or genetically engineered cells (Thompson et al., 2000; Castillo et al., 2008; Nolte et al., 2008; Thompson, 2009; Gernert et al., 2011).

For neural transplantation studies into the basal ganglia in epilepsy research, mainly GABAergic cells were used. The SNr has been of particular interest as target region for transplantation of GABA-producing cells, because it has long been known that pharmacological inhibition of the SNr leads to anticonvulsant effects in different animal models of epilepsy (Iadarola and Gale, 1982; Le Gal La Salle et al., 1983; McNamara et al., 1984; Sperber et al., 1987; De Sarro et al., 1991; Moshé et al., 1992; Depaulis et al., 1994; Dybdal and Gale, 2000; Bröer et al., 2012). Furthermore, considering functional changes in the GABAergic system, the SNr is involved in the development of the epileptic network (Gernert et al., 2004; Töllner et al., 2011). This led to the transplantation of inhibitory GABA-producing cells into the SNr and indeed, significant anticonvulsant effects were reported (Lösch et al., 1998; Thompson et al., 2000; Thompson and Suchomelova, 2004; Castillo et al., 2006; Castillo et al., 2008; Nolte et al., 2008).

Until now, the main problem of all neurotransplantation studies targeting the basal ganglia in epilepsy models is that, independent of the transplanted cell type or utilized seizure or epilepsy models, so far only transient anticonvulsant effects could be induced. To make this strategy clinically applicable as an alternative treatment option for pharmacoresistant patients, long-lasting anticonvulsant effects have to be proved.

1.4.1 Transplantation of Neuronal Precursor Cells into Basal Ganglia in Epilepsy Research

In 1990, Fine et al. were the first who reported a reduced susceptibility for the proconvulsive substance pilocarpine in response to transplantation of fetal GABAergic precursor cells into the SNr. However, this effect was unspecific because it was also observed after transplantation of non-GABAergic cells. The duration of the anticonvulsant effects and the
survival rate of the grafted cells were not determined (Fine et al., 1990). Later, Löscher et al. (1998) proved that grafting fetal, striatal GABAergic cells, isolated from rat fetuses at day 14 of gestation increased afterdischarge thresholds and reduced the seizure severity in the kindling model for temporal lobe epilepsy, when grafted into multiple sites of the SNr. These effects were not detected after transplantation of non-GABAergic control cells or cell medium, thus showing that the observed effects were specific for the GABAergic precursor cells (Löscher et al., 1998).

In a few human epilepsy patients, porcine fetal inhibitory precursor cells were already used in a proof-of-principle neurotransplantation study. Although promising anticonvulsant effects were obtained after grafting into the seizure focus, further studies were not conducted due to the risk for retroviral infections of the patients (Schachter et al., 1998). By using cells from humanized, genetically engineered pigs, this concern, as well as the problem of graft rejection after xenotransplantation, could eventually be resolved. Whether similar anticonvulsant effects can also be observed after transplantation of porcine precursor cells into basal ganglia, is part of the present study.

Apart from inhibitory precursors typically derived from the ganglionic eminence, cells prepared from the whole mesencephalon may be promising candidate cells for grafting in experimental epilepsy (Backofen-Wehrhahn et al., 2014). Ventral mesencephalic cells develop a dopaminergic phenotype and are typically used for cell-replacement therapy in Parkinson’s disease (Armstrong et al., 2003). Wegner and colleagues, however, proved that cells isolated from the whole mesencephalon develop into GABAergic cells during in vitro culturing (Wegner et al., 2008; Wegner et al., 2009). Therefore, cultured mesencephalic precursor cells are a highly interesting cell type for grafting in epilepsy.

1.4.2 Transplantation of GABAergic Cell Lines into the Basal Ganglia in Epilepsy Research

Apart from fetal GABAergic precursor cells (Fine et al., 1990; Löscher et al., 1998), immortalized GABA-producing cells have been used for transplantation into the SNr in experimental epilepsy research. These cell lines were in part additionally genetically engineered to overexpress the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). In a first step, cortical mouse neurons engineered to express GAD were transplanted in rat models for epilepsy (Thompson et al., 2000; Gernert et al., 2002). Since this was a
xenotransplantation from mouse into rat, immortalized inhibitory striatal rat cells (M213-20), in part additionally overexpressing human GAD (hGAD), were later used for neurotransplantation in epilepsy research (Giordano et al., 1993; Conejero-Goldberg et al., 2000; Ross et al., 2002; Castillo et al., 2006; Castillo et al., 2008; Nolte et al., 2008).

Castillo et al. (2006) were the first who reported anticonvulsant effects by grafting hGAD-overexpressing cells into the SNr in kainic acid rat models for temporal lobe epilepsy. A study of our group using the hGAD-overexpressing as well as the GABAergic parental cell line for neurotransplantation into the SNr was the first to also prove an anticonvulsant efficacy of the parental cell line in the amygdala-kindling model of temporal lobe epilepsy (Nolte et al., 2008). The cells induced an increase of the generalized seizure threshold in kindled rats one week after grafting. However, grafting of the hGAD-overexpressing cell line unexpectedly resulted in inflammatory tissue reactions that were not described in previous studies. These reactions were discussed to be most likely due to a combination of host-specific and model-specific factors (Nolte et al., 2008).

1.4.3 The Subthalamic Nucleus as Target Region for Neurotransplantation

Apart from the SNr, the STN is a suitable target region for focal seizure modulation, as was shown by several pharmacological studies (Feger and Robledo, 1991; Deransart et al., 1996; Velisková et al., 1996; Deransart et al., 1998; Dybdal and Gale, 2000; Bröer et al., 2012). In
primates the STN is located ventromedially to the globus pallidus and belongs to the subthalamus of the diencephalon. In the rat, the STN is located dorsal to the capsula interna and the cerebral peduncle and anterior to the SNr (Heise and Mitrofanis, 2004). The STN sends excitatory, glutamatergic input to the SNr. The pharmacological inhibition of the STN, e.g. with muscimol or vigabatrin, has been reported to cause anticonvulsant effects. This anticonvulsant effect is thought to be mediated by an indirect inhibition of the SNr due to reduced excitatory subthalamo-nigral activity (Fig. 3) (Dybdal and Gale, 2000; Deniau et al., 2007; Bröer et al., 2012).

![Schematic illustration](image)

**Fig. 3:** Schematic illustration of mechanisms for focal manipulation of the network between subthalamic nucleus (STN) and substantia nigra pars reticulata (SNr) to induce anticonvulsant effects. The direct inhibition of the SNr by injection of GABAergic drugs or transplantation of GABAergic cells (left), or the indirect inhibition of the SNr by inhibiting the STN with GABAergic drugs and thus reducing the glutamatergic input into the SNr (right), leads to a disinhibition of downstream structures, which mediates anticonvulsant effects. GABA, γ-aminobutyric acid.

Indeed, the focal microinjection of the GABA_A-receptor agonist muscimol into the STN has been shown to reduce the metabolic and electrophysiological activity of the SNr (Feger and Robledo, 1991). Furthermore, microinjection of muscimol into the STN has been reported to reduce the occurrence of acute seizures induced with flurothyl or bicucullin (Velísková et al., 1996; Dybdal and Gale, 2000) and spontaneous non-convulsant seizures in an animal.
model for absence epilepsy (Deransart et al., 1996). Recently, Bröer et al. (2012) demonstrated clear anticonvulsant effects after microinjection of vigabatrin into the STN. The anticonvulsant efficacy of vigabatrin is based on the increased amount of GABA due to an irreversible inhibition of the GABA-degrading enzyme GABA-transaminase (Sabers and Gram, 1992; Treiman, 2001). Moreover, a bilateral microinjection into the STN induced even stronger anticonvulsant effects than microinjections into the SNr or systemic applications of vigabatrin (Bröer et al., 2012). The results indicated that the STN may also be a more promising target region for neural transplantation than the SNr.

Two further reasons are shedding a beneficial light on the use of the STN as a target for neural transplantation in epilepsy research. First, the STN is already clinically established for functional neurosurgery (Benabid, 2007; Al-Otaibi et al., 2011) and second, there is experimental experience in grafting cells into the STN in animal models of Parkinson’s disease (Freeman et al., 2001; Mukhida et al., 2001; Inden et al., 2005; Anderson and Caldwell, 2007; Mukhida et al., 2008; Newman and Bakay, 2008). Nevertheless, until now the STN was not used as target region for neurotransplantation in epilepsy research, which is why I conducted a first proof-of-principle study during my thesis work.

1.5 Immunomodulation to Prevent Graft Rejection

For neurotransplantation as a treatment strategy for different neurological disorders including epilepsies, a wide range of cell types are investigated. Apart from genetically engineered cell lines, especially human and porcine progenitor cells are of interest (Schachter et al., 1998; Shetty, 2011; Hovakimyan et al., 2012; Gernert, 2013). To make this approach applicable for clinical use, the safety and efficacy of grafting different cell types into appropriate brain regions has to be verified in experimental animal models. Xenotransplants, e.g. human or pig derived cells into rodents, have a high risk of rejection within 2-4 weeks by the host’s immune system (Brundin et al., 1985b; Brundin et al., 1988; Ryba et al., 1995; Castilho et al., 2000; Larsson et al., 2000; Wennberg et al., 2001; Kahan, 2009). In order to prevent graft rejections and thereby enable sufficient cell survival and integration into the host tissue, an immunological modulation of the host animals is necessary.
1.5.1 Pharmacological Immunosuppression

Cyclosporine A (CsA) is the commonly used immunosuppressive agent to prevent graft rejections in xenotransplantation studies (Kahan, 1989, 2009; Jensen et al., 2012; Tedesco and Haragsim, 2012; Skardelly et al., 2013). Only few studies indicated that CsA does not affect the survival, integration, or function of human precursor cells grafted into the striatum in an animal model of Parkinson’s disease because of the brain's status as an immunologically privileged organ (Schwarz et al., 2006). Most other studies, however, proved that CsA is necessary to promote long-term survival and functional integration, although it is not sufficient to completely suppress immune responses after grafting porcine precursor cells (Brundin et al., 1985b; Honey and Shen, 1999; Larsson and Widner, 2000; Wennersten et al., 2006).

The immunosuppressive activity of CsA is mediated by a reduction of the T-cell-mediated immune response. CsA binds to the immunophilin cyclophilin A and thereby inhibits the activation of the cytosolic phosphatase calcineurin. This inhibition of calcineurin prevents the transcription of cytokines, such as interleukin-2, γ-interferon, and tumor necrosis factor-α, and consequently also prevents the formation of activated T-lymphocytes (Barten et al., 2007; Cook et al., 2009; Tedesco and Haragsim, 2012). Further mechanisms are discussed to be responsible for the neuroprotective effect of CsA observed in patients treated with CsA after traumatic brain injury. It is assumed that CsA reduces oxidative stress and the release of the apoptosis-inducing factor. By inhibiting calcineurin, CsA also preserves the stability of the cell structure after traumatic brain injuries (Cook et al., 2009; Osman et al., 2011). Additionally to traumatic brain injury, neuroprotective effects of CsA were also observed in stroke, ischemia, Huntington’s disease, and dyskinesia patients and rodent models (Uchino et al., 1998; Ouary et al., 2000; Borlongan et al., 2002; Sinigaglia-Coimbra et al., 2002; Signoretti et al., 2004; Osman et al., 2011). Furthermore, CsA may act neuroprotective by maintaining the mitochondrial membrane potential due to preventing the opening of the mitochondrial permeability transition (MPT) pore (as discussed by Santos and Schauwecker, 2003, and Jung et al., 2012).

The wide range of adverse effects caused by CsA is a major problem of the immunosuppressive treatment. Besides the increased risk of viral, bacterial, and fungal infections, common adverse effects are hypertension, diarrhea, and especially
nephrotoxicity, which can be observed in 25-75% of patients treated with CsA (Cook et al., 2009; Kahan, 2009; Osman et al., 2011; Tedesco and Haragsim, 2012). Furthermore, mild or severe neurotoxic effects could be observed in up to 40% of patients, including tremor, blindness, psychoses, hallucinations, and new onset of seizures (Gijtenbeek et al., 1999; Serkova et al., 2004; Raza et al., 2007). The seizure activities during CsA treatment are often linked to the posterior reversible encephalopathy syndrome, which is a poorly understood clinical syndrome associated with confusion, headache, cortical blindness, visual and motor abnormalities, and seizures (1.5-6% of patients) (Gijtenbeek et al., 1999; Bechstein, 2000; Thompson et al., 2009). Additionally, patients with this syndrome show changes of the white-matter in magnetic resonance imaging (Gijtenbeek et al., 1999; Magnasco et al., 2008). In rodent models, epileptiform activities in the electroencephalography (EEG) and seizures were also observed during CsA treatment (Racusen et al., 1988; Famiglio et al., 1989).

For neurotransplantation studies in experimental epilepsy research, it is therefore necessary to evaluate whether or not the immunosuppression itself influences seizure susceptibility in the models used. Indeed, several studies reported either proconvulsant or anticonvulsant effects by CsA in rodents. In these studies, a high diversity of methods was used, including different seizure and epilepsy models, mice and rat strains, CsA dosages, and application routes, which possibly explain the inconsistent results.

A treatment with CsA for instance increased the intensity of acute seizures induced with bicuculline (Shuto et al., 1999; Fujisaki et al., 2002), pilocarpine (Setkowicz et al., 2004), and pentylenetetrazole (Asanuma et al., 1995b). Furthermore, in electrically induced acute seizures the threshold for seizure induction was lowered (Racusen et al., 1990) and the incidence of seizures was elevated (Yamauchi et al., 2005), indicating a proconvulsant effect. Also, epileptiform activities in the EEG of CsA-treated animals were observed (Racusen et al., 1988; Famiglio et al., 1989). In contrast, anticonvulsant effects are also described. Jung et al. (2012) observed a decrease in the number of seizures per day in CsA-treated mice in a chronic epilepsy model. In acute seizure models, CsA treatment was shown to increase the threshold of pentylenetetrazole-induced seizures and the latency time until the onset of the seizures (Asanuma et al., 1995a; Homayoun et al., 2002). Furthermore, CsA treatment was reported to reduce the intensity of pilocarpine-induced seizures and to elevate the survival
rate of the treated animals (Setkowicz and Ciarach, 2007). Likewise, in vitro studies also showed an increase (Wong and Yamada, 2000) or a decrease (Jung et al., 2012) of epileptiform activity in brain slice recordings, which may depend on the used CsA dosage (Gorji et al., 2002).

1.5.2 Induction of Immunological Tolerance

The principle of neonatal induction of tolerance as an alternative to a chronic pharmacological immunosuppression has been known for a long time and has been conducted with various tissue types (e.g. liver cells, bone marrow cells, spleen cells) for different transplantation approaches (Billingham et al., 1953; Billingham and Brent, 1956; Ando et al., 1991; West et al., 1994a, b; Ridge et al., 1996; Modigliani et al., 1997; Adkins et al., 2004; Peiguo et al., 2012). Kelly and colleagues were the first to develop a strategy to achieve long-term survival of neural tissue grafts based on the induction of tolerance during the neonatal period towards the cells intended for later neurotransplantation (Kelly et al., 2009). In that study, human and mouse primary brain tissue as well as neural precursor cells were used for induction of tolerance and for later transplantation in rats. The survival rate of cells xenografted into neonatally tolerance-induced rats was identical to survival in rats receiving a traditional immunosuppression with CsA. For optimal survival of the xenografts, the tolerance induction had to be done during postnatal day 0-5, otherwise the grafted cells were completely rejected by the host (Kelly et al., 2009). These results could be confirmed by another group using human embryonic mesenchymal stem cells (Zhang et al., 2013). However, other studies reported a failure of this approach. The grafting of human glial-restricted precursor cells or human embryonic cortical tissue in neonatally tolerance-induced mice and the grafting of a human neural stem cell line in neonatally tolerance-induced rats induced tissue reactions that were comparable to the reactions in non-immunomodulated control rats (Janowski et al., 2012; Roberton et al., 2013). Furthermore, an immune-cell infiltration into the transplantation site was detected (Janowski et al., 2012). These results indicate that a neonatal induction of tolerance is not universally applicable and may depend on the donor tissue used for tolerance-induction and subsequent grafting or on the host species. Further studies should investigate which properties the donor cells need to have in order to successfully induce immunological tolerance of the host species.
1.6 Timed Intravenous Pentylenetetrazole Seizure Threshold Test and Amygdala-Kindling Model

To evaluate putative effects of neurotransplantation and immunomodulation on seizure thresholds, two different rat models were used. As an acute seizure model, the pentylenetetrazole (PTZ) seizure threshold test was used in the studies presented here. Pentylenetetrazole is a GABA<sub>A</sub>-receptor-antagonist, which binds to the picrotoxin binding site of the α-subunit of the GABA<sub>A</sub>-receptor. Since the GABA<sub>A</sub>-receptor is a chloride ion channel, PTZ prevents postsynaptic hyperpolarization and counteracts the GABAergic inhibition. High dosages of PTZ induce seizures, whereas subconvulsive dosages lower the seizure threshold (Cremer et al., 2009; Löscher, 2009).

For an experimental induction of an acute, generalized seizure, PTZ is administered intravenously using an infusion pump system (Fig. 4). This leads to the occurrence of different seizure types (myoclonic twitches followed by clonic and tonic seizures), which are used as end-points in this model for stopping the infusion. The individual seizure threshold dose to trigger the respective end-point can then be determined for each animal.

As the effect of PTZ is mediated via the GABA<sub>A</sub>-receptor, this model is especially sensitive for manipulations of the GABAergic system. Furthermore, this test is a fast screening model to investigate putative anti- or proconvulsant effects of tested substances or GABA-producing cells (Löscher, 2009).

As a chronic epilepsy model, we used the electrical amygdala-kindling model, in which repeated (daily) stimulations of the amygdala cause seizures, which progressively increase in severity and duration (Goddard et al., 1969). Fully kindled rats are then characterized by permanent changes in brain function resulting in a chronically higher seizure susceptibility.
In these fully kindled rats, seizure thresholds can be repeatedly determined to evaluate pro- or anticonvulsant effects in response to cell grafting or pharmacological challenge.

1.7 Aim of the Studies

1.7.1 Study 1: Grafting GABAergic Cells into the Subthalamic Nucleus

Neural transplantation of inhibitory cells into the basal ganglia, known to be involved in seizure modulation, is one promising experimental approach to overcome the problem of pharmacoresistant epilepsies. In experimental epilepsy models, it could be repeatedly shown that transplantations of genetically engineered GABA-producing cells or GABAergic precursor cells into the SNr act anticonvulsant (Löscher et al., 2008; Al-Otaibi et al., 2011). A recent pharmacological study of our group showed that the STN might even be a more promising target region for focal seizure manipulations (Bröer et al., 2012). Nevertheless, there have been no studies using the STN as target region for neurotransplantation in epilepsy research. GABA-producing cell lines were now transplanted for the first time into the STN in experimental research. The aim was to establish a first proof-of-principle study evaluating putative anticonvulsant effects in response to grafting inhibitory cells into the STN.

The working hypotheses were:

1. The transplantation of GABA-producing cell lines into the STN of rats causes anticonvulsant effects in an acute seizure model.

2. Similar to the findings from a recent pharmacological study (Bröer et al., 2012), the anticonvulsant effects are stronger and more long-lasting than previous results obtained by a transplantation of the same cell lines into the SNr using a different epilepsy model (Nolte et al., 2008).

3. The anticonvulsant effects are specific for inhibitory cells and for the STN as transplantation site.

4. These cell lines grafted into the STN are not rejected and tumor formations can be excluded.
1.7.2 Study 2: Effects of Cyclosporine A on Seizure Thresholds

For clinical translation, human or porcine donor cells are more relevant than rodent cell lines and have to be evaluated concerning anticonvulsant efficacy and safety in preclinical animal models. For these xenotransplantation studies, graft rejection has to be prevented by appropriate immunosuppression. In the second study, we thus evaluated the effect of a pharmacological immunosuppression with CsA on acute and chronic seizure thresholds in rats. Earlier studies showed inconsistent results in terms of anticonvulsant or proconvulsant effects by CsA in various experimental epilepsy models and species (Famiglio et al., 1989; Fujisaki et al., 2002; Homayoun et al., 2002; Setkowicz et al., 2004; Jung et al., 2012). The aim was to identify a suitable immunosuppression protocol that can be used for xenotransplantations without influencing the respective models and without having severe adverse effects for the animals. Therefore, we comparably investigated different CsA doses, application routes, preparations, and treatment durations.

The working hypotheses were:

1. It is possible to establish a CsA immunosuppression protocol that does not influence seizure thresholds in the intravenous PTZ seizure threshold test and in the amygdala-kindling model of epilepsy.
2. The ideal protocol should not affect behavior, locomotion, and physiological measures of the treated rats.

1.7.3 Study 3: Porcine Cell Grafting and Induction of Tolerance

In the third study, mesencephalic pNPCs were used for transplantation into the STN of rats. To prevent a rejection of the xenografts and to compare different immunomodulatory strategies with respect to their abilities to promote survival and long-term anticonvulsant effects of the grafts, rats either received a pharmacological immunosuppression with CsA or were neonatally tolerance-induced for the later transplanted cells (Kelly et al., 2009). Rats without immunomodulation served as controls. We wanted to evaluate, which immunomodulation strategy is more suited for xenotransplantations into the STN. In other words, we investigated which strategy better promotes the survival and putative anticonvulsant effects of the porcine cells, and at the same time has less adverse effects.
The working hypotheses were:

1. Transplantation of pNPCs into the STN of neonatally tolerance-induced rats induces long-term anticonvulsant effects.

2. The transplantation into rats receiving a pharmacological immunosuppression also induces anticonvulsant effects which, in contrast, are only transient and disappear after discontinuation of CsA treatment.

3. Grafting of the porcine cells into rats that were not immunomodulated induces graft rejection and, therefore, at the best causes only transient anticonvulsant effects.

4. In contrast to the pharmacological immunosuppression with CsA, the induction of immunological tolerance has no adverse effects for the rats.
2 Anticonvulsant Effects by Bilateral and Unilateral Transplantation of GABA-producing Cells into the Subthalamic Nucleus in an Acute Seizure Model

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D) Analysis of experiments: A.H., M.G.
E) Writing of manuscript: M.G., A.H.
Abstract

Neural transplantation of GABA-producing cells into key structures within seizure-suppressing circuits holds promise for medication-resistant epilepsy patients not eligible for resection of the epileptic focus. The substantia nigra pars reticulata (SNr), a basal ganglia output structure, is well known to modulate different seizure types. A recent microinjection study by our group indicated that the subthalamic nucleus (STN), which critically regulates nigral activity, might be a more promising target for focal therapy in epilepsies than the SNr. As a proof of principle, we therefore assessed the anticonvulsant efficacy of bilateral and unilateral allografting of GABA-producing cell lines into the STN using the timed intravenous pentylenetetrazole seizure threshold test, which allows repeated seizure threshold determinations in individual rats. We observed (a) that grafted cells survived up to the end of the experiments, (b) that anticonvulsant effects can be induced by bilateral transplantation into the STN using immortalized GABAergic cells derived from the rat embryonic striatum and cells additionally transfected to obtain higher GABA synthesis than the parent cell line, and (c) that anticonvulsant effects were observed even after unilateral transplantation into the STN. Neither grafting of control cells nor transplantation outside the STN induced anticonvulsant effects, emphasizing the site and cell specificity of the observed anticonvulsant effects. To our knowledge, the present study is the first showing anticonvulsant effects by grafting of GABA-producing cells into the STN. The STN can be considered a highly promising target region for modulation of seizure circuits and, moreover, has the advantage of being clinically established for functional neurosurgery.
3 Comprehensive Study of Acute and Chronic Treatment with Different Preparations, Doses, and Administration Routes of Cyclosporine A on Seizure Thresholds and Adverse Effects

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3.1 Abstract

Neuronal transplantation is a promising experimental treatment approach for intractable epilepsies, but rejection of porcine or human cells in rodent epilepsy models requires adequate immunosuppression to enable long-term survival of xenografts. The commonly used immunosuppressive drug cyclosporine A (CsA) itself was suggested to affect seizure thresholds. However, putative pro- or anticonvulsant effects of CsA have not yet been sufficiently explored in a direct comparison study involving different preparations, dosages, and application routes. We therefore comprehensively investigated the effects of acute versus chronic treatment using different dosages (5 mg/kg, 10 mg/kg), application routes (i.p., s.c.), and preparations of CsA (pure substance solved in polyethoxylated castor oil and a ready-to-use drug additionally containing ethanol) on seizure thresholds in rats in the pentylenetetrazole seizure threshold test and the amygdala-kindling model for temporal lobe epilepsy. None of the CsA treatment regimens induced acute changes of seizure thresholds. Chronic CsA treatment also did not robustly change seizure thresholds. The absorption of i.p. applied CsA in the ready-to-use preparation significantly exceeded the absorption from s.c. injection and from the pure CsA preparation as evaluated by whole blood analyses. Observed adverse effects differed between CsA treatment regimens and included reversible diarrhea, lowered body temperature, and tremor, the latter two of which were also induced by vehicle injections containing ethanol and/or polyethoxylated castor oil. Our data suggest that chronic treatment (2 weeks) with 10 mg/kg CsA injected i.p. in the ready-to-use preparation is an appropriate protocol for use in neural transplantation in epilepsy research applying the presently used rat models. Transplantation studies in experimental epilepsy research require a careful assessment of putative CsA effects on seizure thresholds in the specific experimental settings to be used.

Key words: Epilepsy, kindling, pentylenetetrazole, immunosuppression, transplantation, xenograft

3.2 Introduction

Neural transplantation is a promising treatment strategy for intractable epilepsies and other neurological disorders. Cells from different sources, especially human or porcine donor cells, have been used in preclinical transplantation studies to replace damaged or lost brain cells
or to compensate for imbalances in excitation and inhibition (Schachter et al., 1998, Brevig et al., 2000). Before clinical translation, the cells must first be tested preclinically in rodent models to demonstrate and refine safety and efficacy after grafting into appropriate brain targets. Although the brain is characterized by partial immune privilege, xenogeneic cells grafted into the brain are typically rejected and thus an immunosuppression is required to permit graft survival (Brundin et al., 1985, Brundin et al., 1989, Pakzaban and Isacson, 1994, Honey and Shen, 1999, Wennberg et al., 2001, Borlongan and Sanberg, 2002, Irons et al., 2004, Wennersten et al., 2006, Rota Nodari et al., 2010, Jensen et al., 2012).

Cyclosporine A (CsA), a potent immunosuppressive agent (Borel, 1980, Kahan, 1989, Borel and Kis, 1991, Kahan, 2009), is commonly used alone or in combination with other drugs to prevent xenograft rejections in experimental transplantation studies in rodents. It is of pivotal importance, that the used CsA treatment routine does not affect seizure parameters itself, which are to be investigated to demonstrate efficacy and safety of cell transplantation in experimental epilepsy studies.

However, apart from its known mechanisms of action to lower T cell-mediated immune responses (Hess and Colombani, 1986, Drugge and Handschumacher, 1988, Santori et al., 1997), CsA appears to paradoxically mediate both neuroprotection (Sullivan et al., 2000, Santos and Schauwecker, 2003, Cook et al., 2009, Osman et al., 2011, Uchino et al., 2013, Yokobori et al., 2013) and neurotoxicity (Hauben, 1996, Gijtenbeek et al., 1999, Bechstein, 2000, Serkova et al., 2004) across the same range of drug concentration. Neurotoxic adverse effects may present as new onset seizures caused by acute encephalopathy. The occurrence of seizures, which occasionally even persist after CsA discontinuation, has been described upon chronic treatment with CsA in humans (Gleeson et al., 1998, Trullemans et al., 2001, Faraci et al., 2002, Faraci et al., 2003, Gaggero et al., 2006, Navarro et al., 2010) as well as rodents (Racusen et al., 1988, Famiglio et al., 1989).

Both the neurotoxic and the neuroprotective properties thus suggest an impact of CsA on seizure susceptibility, albeit with different directions. Indeed, contrary results are available from in vivo investigations in rodents regarding the modulation of seizure susceptibility by CsA. Some studies revealed proconvulsant effects (Racusen et al., 1988, Famiglio et al., 1989, Racusen et al., 1990, Asanuma et al., 1995b, Shuto et al., 1999, Fujisaki et al., 2002, Setkowicz et al., 2004, Yamauchi et al., 2005), while others showed anticonvulsant effects by
CsA treatment (Moia et al., 1994, Asanuma et al., 1995a, Homayoun et al., 2002, Setkowicz and Ciarach, 2007, Jung et al., 2012). Similarly, several in vitro studies also revealed either an initiation or increase (Wong and Yamada, 2000, Gorji et al., 2002) or a decrease (Jung et al., 2012) of epileptiform activity in brain slice recordings in response to CsA. In general, the contrary results are probably related to the distinct diversity in experimental settings of these studies, but a direct comparison is hampered by the fact that different seizure or epilepsy models, CsA preparations, acute or chronic CsA treatment regimens, dosages, application routes, vehicles, and different rat or mouse strains were used. Furthermore, in none of these studies were CsA whole blood levels investigated to verify correct drug injections.

Therefore, we now performed a comprehensive study of acute and chronic treatments with different preparations, doses, and administration routes of CsA on different experimentally induced seizure thresholds to allow a direct comparison of the different treatment regimens. We sought to identify a treatment strategy that may be amenable for future xenograft studies in rat models of epilepsy. To assess further factors, which may affect seizure parameters by CsA treatment, we determined whole blood levels of the immunosuppressant and included the investigation of CsA-induced changes of behavior and physiological measures.

3.3 Experimental Procedures

The effects of CsA on seizure thresholds were investigated using two different rat models: (A) The timed intravenous pentylenetetrazole (PTZ, metrazole) seizure threshold test (Löscher, 2009), which is an acute seizure model, and (B) the electrical amygdala-kindling model (Goddard et al., 1969), which is considered a chronic epilepsy model. Overviews of the respective study design and sample sizes are given in Fig. 1 and 2.

3.3.1 Animals

To allow comparison with our previous data, adult female Wistar rats (Harlan-Laboratories, Horst, Netherlands) were purchased at a body weight of 200–220 g. Rats used for the PTZ study were housed in groups of three to five, rats used for the kindling study were housed individually after surgical implantation of the kindling electrode. All rats were housed
without males in order to keep them acyclic or asynchronous with respect to their estrous cycle (Kücker et al., 2010). All rats were kept under controlled environmental conditions with a 12-h light/dark cycle (lights on at 6:00 a.m.) for at least one week before onset of experiments. Standard laboratory chow (Altromin 1324 standard diet) and tap water were allowed ad libitum. All animal experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and were formally approved by the animal subjects review board of our institution. All efforts were made to minimize the number of animals used and their suffering.

3.3.2 Timed Intravenous PTZ Seizure Threshold Test

To determine acute seizure thresholds before, at different times during, and after CsA or vehicle treatment, altogether 97 rats (Fig. 2A) underwent the timed intravenous PTZ seizure threshold test as described in detail previously (Löscher, 2009, Rattka et al., 2011, Bröer et al., 2012). Briefly, a 0.8% solution of PTZ (Caesar & Loretz, Hilden, Germany) in saline was infused via a 24-gauge needle (Terumo® Europe n.V., Leuven, Belgium) into the lateral tail vein of the conscious, unrestricted rat. The needle was secured to the tail vein by a piece of adhesive tape and was connected to a syringe by a flexible polyethylene tubing (Kleinfeld Labortechnik, Gehrden, Germany). The PTZ solution was infused using an infusion pump (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA, USA). The infusion was terminated immediately following the onset of the first clonic seizure. The PTZ seizure threshold was calculated in mg PTZ per kg body weight based on the time needed to induce this clonus, the body weight of the animal, the rate of PTZ infusion (1.0 ml/min), and the PTZ concentration. Additionally, we calculated the threshold for the first myoclonic twitch, which always preceded the clonic seizure. All seizures or other abnormal behaviors occurring after this endpoint were noted. For this purpose, rats were closely observed until they resumed normal behavior, which typically was about 1–2 h after PTZ infusion. The intravenous PTZ test can be repeatedly performed in the same rat at intervals of about 48 h, allowing the study of changes in threshold over time (Löscher, 2009). To prevent a kindling effect, in the present study the number of PTZ seizure threshold determinations was limited to a maximum of five in each rat, with intervals of at least one week between testing. Not all time points were assessed in each rat.
3.3.3 Implantation of Kindling Electrode and Kindling

To determine chronic (kindled) seizure thresholds before, at different times during, and after CsA or vehicle treatment, 15 rats underwent electrical kindling via the amygdala followed by seizure threshold determination in fully kindled rats as described earlier (Gernert and Löscher, 2001, Töllner et al., 2011). For implantation of the kindling electrode, rats were anesthetized with isoflurane (CP-Pharma, Burgdorf, Germany; induction 3%, maintenance 1.5%) via an inhalation mask adjusted to a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). In addition, the scalp (2% tetracaine hydrochloride, Caesar & Loretz) and the periost (0.25% bupivacaine hydrochloride; Carbostesin®, AstraZeneca, Wedel, Germany) were locally anesthetized. A teflon-insulated bipolar stainless steel electrode was stereotaxically implanted for stimulation of the right basolateral amygdala and recording of electrographic seizure activity (afterdischarges). The stereotaxic coordinates in millimeters relative to bregma according to the atlas of Paxinos and Watson (2007) were: posterior 2.2 mm, right 4.8 mm, ventral 8.5 mm. The incisor bar was set at −3.3 mm. A stainless steel screw, placed above the left parietal cortex, served as the grounding electrode. Bipolar and ground electrodes were connected to plugs. Additional skull screws and dental acrylic cement were used to anchor the headset. Two days before and until five days after surgery, rats were treated twice daily with the antibiotic marbofloxacin (3 mg/kg s.c.; Marbocyl® FD, WDT, Garbsen, Germany).

After a post-surgical recovery of at least two weeks, rats were kindled once daily with a constant current stimulation (500 μA, 1 msec monophasic square-wave pulses at 50 Hz, 1 sec train) until at least 10 generalized kindled seizures classified as stage five (see below) according to Racine (1972) were elicited (fully kindled rats). One day before and at the earliest 3 days after kindling, the initial (pre-kindling) and the first post-kindling stimulation threshold for eliciting afterdischarges in the electroencephalogram (ADT, afterdischarge threshold) were determined for each animal using an ascending stair step procedure as described previously (Gernert and Löscher, 2001). Therefore, rats were stimulated starting from a current intensity of 30 μA (other parameters as during kindling), followed by increases in current intensity by about 20% of the previous current at intervals of 1 min until afterdischarges of at least 3 sec duration were elicited. Further post-kindling ADTs were determined twice a week, again using the ascending stair step procedure, but starting from a
current intensity of three 20% steps below the respective preceding ADT, until rats exhibited reproducible ADTs. In addition to the ADTs, the thresholds for eliciting secondarily generalized motor seizures (GST, generalized seizure threshold; stage 4 or 5 seizures according to Racine (1972) were determined for each kindled rat. Usually, fully kindled rats show generalized seizures at the ADT, so that the ADT equals the GST. Otherwise, the current was further elevated up to the GST. Apart from the seizure thresholds themselves, further parameters were recorded during kindling and during ADT and GST determination: seizure severity (SS), seizure duration (SD), and afterdischarge duration (ADD). During GST determination, the latency period from stimulation until occurrence of secondarily generalized motor seizure (LP) was additionally determined. SS was classified behaviorally according to Racine (1972): stage 1, immobility, slight facial clonus (eye closure, twitching of vibrissae, sniffing); stage 2, head nodding associated with more severe facial clonus; stage 3, clonus of one forelimb; stage 4, rearing, often accompanied by bilateral forelimb clonus; stage 5, tonic-clonic seizure accompanied by loss of balance and falling. SD was the time from beginning of electrical stimulation until the end of motor seizures. ADD was the total duration of EEG spikes with amplitudes of at least twice the amplitude of the prestimulus recording and a frequency of at least 1/s, including the time of stimulation.

3.3.4 CsA Treatment Regimens

For an overview of the detailed group design and the sample sizes see Figs. 1 and 2. Two different CsA preparations were used in the present study and compared regarding their putative effects on seizure thresholds and behavior: The pure substance CsA (Sigma-Aldrich, pCsA) and a dilution of the injection solution of the ready-to-use drug Sandimmune® (50 mg/ml, Novartis Pharmaceuticals). Additionally, the influence of different application routes, intraperitoneally (i.p.; Injection volume, 3 ml/kg) and subcutaneously (s.c.; Injection volume, 1 ml/kg), and two dosages known to be sufficiently immunosuppressant for intracerebral xenograftings in rats, 5 mg/kg and 10 mg/kg (e.g., Duan et al., 1996, Larsson et al., 2001, Jensen et al., 2012), were comparably investigated. The pure substance pCsA was solved in 20% Cremophor® EL (polyethoxylated castor oil) in saline to increase solubility of CsA (Ran et al., 2001). The ready-to-use drug Sandimmune® was diluted in saline and contained in the final concentrations not only Cremophor® EL (5 mg/kg i.p., 2.17%; 5 mg/kg s.c., 6.5%; 10
mg/kg i.p., 4.33%; 10 mg/kg s.c., 13%) but also ethanol (5 mg/kg i.p., 1.15%; 5 mg/kg s.c., 3.44%; 10 mg/kg i.p., 2.29%; 10 mg/kg s.c., 6.88%). The absolute amount of i.p. and s.c. applied Cremophor® EL and ethanol is identical within each Sandimmune® dosage, because different injection volumes have been used for i.p. and s.c. applications. Animals receiving the vehicle of pCsA or Sandimmune® (of the 10 mg/kg preparations) and naïve rats served as controls.

Figure 1: Time line illustrating the sequence of procedures during the present study to evaluate the effects of cyclosporine A (CsA) on seizure thresholds in (A) the pentylenetetrazole (PTZ) seizure threshold test and (B) the kindling model, respectively. Animals were treated over a period of 15 days with different CsA preparations or respective vehicles and via different application routes. Seizure thresholds were repeatedly determined at several time points before, during, and after CsA treatment. ADT, afterdischarge threshold; i.p., intraperitoneally; pCsA, pure substance CsA; post, post-treatment; s.c., subcutaneously; wk, week.
3.3.5 CsA Treatment in the PTZ Model

The pre-drug control PTZ seizure threshold was determined one week before starting CsA treatment. Subsequently, rats were randomly assigned to the different treatment groups (Fig. 2A). PTZ seizure thresholds were then determined after the first injection of CsA or vehicle (acute), after one and two weeks of treatment (1 and 2 weeks), and one week after discontinuation of the treatment (1 week post) (Fig. 1A). PTZ seizure thresholds were always determined two hours after injecting vehicle or CsA, i.e. at around peak blood levels of CsA in rats after i.p. application (Luke et al., 1990, Ibarra et al., 1996) (Fig. 2A). This time point
was immediately after behavioral testing and physiological measurements (described below). Seizure threshold determination was performed at the same time of the day (between 9:00 and 12:00 a.m.) to avoid intraday variances. The experimenter was blinded with respect to the treatment regimen.

3.3.6 **CsA Treatment in the Kindling Model**

In the chronic kindling model, only the Sandimmune® preparation of CsA in a dose of 10 mg/kg i.p. was used to confirm observations made in the acute PTZ model. Vehicle-treated kindled rats served as controls. The investigation was designed as a cross-over study (Fig. 1B). After reproducible ADTs were obtained in fully kindled rats, two pre-drug control ADTs and further kindled seizure parameters (as described above) were determined (day -7 and -4). Rats were then treated with either 10 mg/kg CsA in the Sandimmune® preparation or with vehicle via daily i.p. injection (day 1 to day 15), followed by an one week wash-out period and another week to again determine the individual pre-drug control ADTs and further kindled seizure parameters. Subsequently, treatment groups were changed (Fig. 1B). During the entire study period, ADTs and further kindled seizure parameters were constantly determined twice a week, starting from a current intensity three 20% steps below the individual control ADT. ADTs and further kindled seizure parameters were determined 2 hrs after drug or vehicle injection. For calculation of the effect of CsA on kindled seizure parameters, the individual mean of two pre-drug control ADTs (and further kindled seizure parameters) was set to 100% and compared with the respective values during treatment and wash-out. Rats without reproducible pre-drug ADTs and rats, which lost their headset before at least one treatment period was terminated, were excluded from final analysis, so that the initial group size of 15 was reduced to 7 (drug) and 6 (vehicle) during the course of the kindling study (Fig. 2B).

3.3.7 **Blood Sampling and Whole Blood Drug Analysis**

CsA analysis was performed on whole blood samples, which were taken from rats of the PTZ study. Following determination of PTZ seizure thresholds and 2.5 hrs after injection of the respective CsA preparation, blood samples were withdrawn by retrobulbar venous plexus puncture after local anesthesia with tetracaine hydrochloride (2%) for drug analysis in whole
blood. Blood samples were anticoagulated with EDTA (5 mmol/ml whole blood), centrifuged for 2.5 min at 12,000 rpm, and stored at −80°C until analysis. CsA concentrations were measured in the lab of Dr. Grote-Koska (Institute for Clinical Chemistry, Hannover Medical School, Germany) using liquid chromatography coupled with mass spectrometry (LC-MS/MS). Following injection of the different preparations, whole blood CsA levels were typically higher than the immunosuppressant concentration of 200 ng/ml in rats (Lai et al., 1987, Zijlstra et al., 2009). Seizure thresholds and physiological as well as behavioral data from time points at which whole blood CsA level was below 200 ng/ml were excluded from final analyses.

3.3.8 Behavioral Testing Battery and Physiological Measures

To evaluate adverse effects of the different CsA treatment regimens, a set of behavioral tests and physiological measures were carried out on rats of the PTZ study. Tests were performed always immediately before PTZ seizure thresholds were determined and additionally up to two weeks after discontinuation of the treatment to detect putative long-term adverse effects (Fig. 1).

Starting 1 ¾ hrs after CsA injection, rats were placed individually in the center of a round, black-colored open field (80 cm diameter, 25 cm height, 0.4 cm wall thickness) made of polyethylene. Rats were observed for two minutes without disturbance, before the following behaviors were rated using a score adapted from Hönack and Löscher (1995): Hypolocomotion (0, not observable; 1, tendency towards decreased locomotion; 2, clearly decreased locomotion with many breaks; 3, no forward movement), hyperlocomotion (0, not observable; 1, tendency towards increased locomotion; 2, clearly increased locomotion; 3, highly increased locomotion and hectic movements without any breaks), ataxia (0, not observable; 1, slight ataxia with weak stagger; 2, clear ataxia with loss of balance during forward movement; 3, permanent loss of righting reflex despite the attempt to move forward), and tremor, head swaying, circling, stereotyped sniffing, straub tail, piloerection, retracted flanks, and flat body posture (0, not observable; 1, suspicious; 2, observable; 3 intense).

Subsequently, the hyperexcitability test (Moser et al., 1988) was carried out to detect group differences in behavioral excitability and sensory responsiveness that may influence seizure
thresholds. In the approach-response test, a pen held vertically is moved slowly towards the nose of the rat. The response was scored as 1, no reaction; 2, sniffing at the pen; 3, moving away from the pen; 4, freezing; 5, jumping away from the pen; 6, attacking the pen. In the touch-response test, the rat is gently touched at the rump with the blunt end of a pen (response score: 1, no reaction; 2, turning slowly towards the pen; 3, moving away from the pen; 4, freezing; 5, jerking around towards the touch; 6, turning away from the touch; 7, bouncing with or without vocalization). In the finger snap test, a standardized click noise is triggered above the back of the rat (response score: 1, no reaction; 2, normal reaction (moderate bouncing, freezing, flinching, or ear flicking); 3, intense bouncing). In the pick-up test, the rat is picked up by grasping around the body (response score: 1, very easy; 2, easy with vocalization; 3, rearing and turning towards the hand; 4, avoiding the hand and trying to escape; 5, intense bouncing; 6, attacking the hand). Finally, the abdominal muscle tone was tested by palpating the abdomen of the rat (0, decreased; 1, normal; 2, increased).

As physiological measures, the number of solid feces boli was counted, the consistency of the feces was rated (0, solid; 1, soft; 2, diarrhea), the rectal body temperature was measured, and the body weight was determined. Behavioral tests and physiological measures were done in a blinded manner with respect to the treatment of the animals and were done at the same time of the day (between 9:00 and 12:00 a.m.) to avoid intraday variance between animals.

3.3.9 Histological Verification of Kindling Site

After termination of kindling experiments, all kindled rats were deeply anesthetized with chloral hydrate and transcardially perfused with 0.9% saline in 0.01 M phosphate buffer (PBS, pH 7.6), followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.6). The brains were removed, cryoprotected in 30% sucrose in 0.1 M PBS (pH 7.6) and stored at 4 °C. Coronal sections were cut at 40 μm on a freezing microtome. Every second section was Nissl-stained with thionine for verification of stimulation site. Only rats with correct placement of the kindling electrode were included into evaluation of kindling data.
3.3.10 Statistics

Statistical analyses of data were performed depending on whether data were parametric or non-parametric. As verified with the Kolmogorov-Smirnov and the D’Agostino and Pearson tests, parametric data of whole blood CsA levels, relative body temperature, body weight gain, PTZ seizure thresholds, and kindling parameters (except seizure severity) were normally distributed. These data were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni posthoc test to detect putative intergroup differences at each time point and to detect differences between the time points within each treatment group (time points were compared to pre-drug control). Because of missing data at single time points, we did not use paired tests or repeated measurement analyses for time series comparisons. For non-parametric data of behavioral and physiological scores as well as kindled seizure severity, the time series were analyzed using Kruskal–Wallis ANOVA or Friedman test, depending on whether the data sets contained missing values or not. Dunn´s multiple comparisons served as posthoc test for time series analyses. To detect intergroup differences in seizure severity in the kindling model, the Mann–Whitney U-test was used. To verify that the pre-drug control values of body temperature, body weight, PTZ seizure thresholds did not differ between the randomly assigned treatment groups, one-way ANOVA followed by Dunnett’s test for multiple comparisons was used. For comparison of pre-drug control values of kindled seizure parameters between vehicle- and Sandimmune®-treated rats, the t-test or, for seizure severity, the Mann-Whitney U-test was used. For comparison of pre-drug control values of kindled seizure parameters before and after cross-over, the paired t-test or, for seizure severity, the Wilcoxon matched pairs test was used. All statistical analyses were performed with the Prism 5.03 software from GraphPad (La Jolla, CA, USA). All tests were used two-tailed and a value of p ≤ 0.05 was considered significant.

3.4 Results

3.4.1 CsA Whole Blood Concentration

Taken together, the following statistically valid results were obtained with respect to whole blood CsA levels measured in samples taken 2.5 hrs after injection of the different dosages of pCsA and Sandimmune®: (a) chronic treatment > acute treatment, (b) Sandimmune® > pCsA, (c) i.p. > s.c., (d) high dosages > low dosages (Fig. 3A).
**Figure 3:** Effects of treatment with different CsA preparations and application routes on whole blood CsA levels, relative body temperature, and body weight gain. (A) Means ± SEM of whole blood CsA levels showing significant differences over time (*p<0.05; **p<0.001) and between treatment groups (*p<0.05; **p<0.01; ***p<0.001). Sample sizes are given within the bars. Whole blood CsA levels of pCsA- and high-dose Sandimmune®-treated rats significantly increased during one week chronic treatment compared to acute injection and then remained stable during further treatment. Note that rats treated with Sandimmune® 10 mg/kg i.p. reached significantly higher whole blood CsA levels than rats treated with pCsA 10 mg/kg i.p. and also higher levels than rats treated with Sandimmune® 10 mg/kg s.c.
Whole blood CsA levels typically accumulated significantly during the course of the treatment. Regardless of the dosage, whole blood CsA levels significantly increased after one week of treatment with pCsA compared to acute injection (p < 0.05). Comparably, whole blood CsA levels significantly increased after one and two weeks of treatment with Sandimmune® 10 mg/kg, regardless of the application route, compared to acute injection (p < 0.001). There were no differences between one and two weeks of treatment, showing that whole blood CsA levels reached a plateau during chronic treatment. With respect to the CsA preparation, rats treated with Sandimmune® 10 mg/kg i.p. reached significantly higher whole blood CsA levels than rats treated with pCsA 10 mg/kg i.p. (p < 0.05 after 1 week and p < 0.001 after two weeks treatment). With respect to the application route, i.p. injection of Sandimmune® resulted in significantly higher whole blood CsA levels than s.c. injection of Sandimmune® (p < 0.001 for 10 mg/kg at any time point and p < 0.01 and p < 0.05 for 5 mg/kg after one and two weeks, respectively). Finally, as expected, the high dosage of 10 mg/kg typically caused higher whole blood CsA levels than the lower dosage of 5 mg/kg. This is especially obvious after chronic treatment (for details refer to Fig. 3A). Taken together, the highest whole blood CsA levels were reached after i.p. injection of Sandimmune® 10 mg/kg (acute: 2092.44 ± 79.36 ng/ml; 1 week: 3321.44 ± 272.12 ng/ml; 2 weeks: 4082.33 ± 454.85 ng/ml). The lowest whole blood CsA level was reached after acute s.c. injection of
Sandimmune® 5 mg/kg (406.2 ± 38.34 ng/ml), because pCsA was not applied by s.c. injection. Noteworthy, the whole blood CsA levels did not correlate with changes in seizure thresholds (not illustrated).

3.4.2 Basal (pre-drug) PTZ Seizure Thresholds

During pre-drug control seizure threshold determination, intravenously applied PTZ in an amount of 18.47 ± 0.31 mg/kg (mean ± SEM) was necessary to induce myoclonic twitches (range 11.18 to 25.54 mg/kg) and 21.58 ± 0.35 mg/kg was necessary to induce clonic seizures (range 13.64 to 34.50 mg/kg). Although one-way ANOVA indicated differences in the pre-drug control PTZ thresholds for induction of myoclonic twitches (p = 0.0179), posthoc comparisons with the Bonferroni method did not show significant differences between treatment groups (naïve rats, vehicle-treated rats, and rats treated with different CsA preparations, dosages, and application routes). For the pre-drug control PTZ clonic seizure thresholds, one-way ANOVA did not indicate differences between treatment groups (p = 0.0927).

3.4.3 Lack of Robust Acute and Chronic Effects of CsA on PTZ Seizure Thresholds

Taken together, neither pCsA nor Sandimmune® caused robust changes of PTZ seizure thresholds. Independent of dosage and application route, none of the tested CsA preparations caused acute changes in PTZ seizure thresholds 2 hrs after a single drug injection, neither compared to pre-drug values nor compared to naïve and vehicle-treated rats (Fig. 4). Concerning chronic CsA treatment up to two weeks, only subtle effects on PTZ seizure thresholds were observed. I.p. injection of pCsA did not change PTZ seizure thresholds over time. However, differences between treatment groups were indicated by two-way ANOVA (p = 0.0122) for clonic seizure thresholds in the pCsA treatment group. The clonic seizure threshold was significantly lower in rats one week after termination of treatment with 10 mg/kg pCsA compared to vehicle-treated rats (p < 0.05; Fig. 4A), but not compared to naïve rats.

Although two-way ANOVA indicated significant changes of seizure thresholds over time in response to i.p. (p = 0.0325 for myoclonic twitch and p = 0.0034 for clonic seizure) and s.c. (p = 0.0014 for myoclonic twitch and p = 0.0040 for clonic seizure) injection of
Sandimmune®, posthoc analysis revealed a significant increase only for the clonic seizure threshold after two weeks of daily i.p. injection of 5 mg/kg Sandimmune® compared to the pre-drug seizure threshold (p < 0.01; Fig. 4B). However, the clonic seizure threshold after two weeks of daily i.p. injection of 5 mg/kg Sandimmune® did not differ significantly from the respective seizure thresholds in naïve and vehicle-treated rats, respectively (Fig. 4B and 4C).

**Figure 4:** Pentylenetetrazole (PTZ) seizure thresholds over time shown as means and SEM relative to pre-drug control. Thresholds for induction of first myoclonic twitch (left column) and clonic seizures (right column) were determined at different time points before (pre-drug), during (acute, 1 week (1wk), 2 weeks (2wk)) and after treatment (1 wk post). Rats received two different dosages of the pure substance cyclosporine A (pCsA) intraperitoneally (A), or of the ready-to-use drug Sandimmune® (Sand) intraperitoneally (B) or subcutaneously (C). Naïve and vehicle-treated rats served as controls. Sample sizes are given within the bars. In general, no robust effects on seizure thresholds were detected, although some isolated significant intergroup (*p<0.05) and time series (**p<0.01) changes occurred as indicated in the figure.
3.4.4 Kindling Development and Basal (pre-drug) Kindling Parameters

In kindled rats receiving either Sandimmune® or vehicle, 6.4 ± 0.7 (mean ± SEM) stimulations were needed until the first secondary generalized stage 5 seizure occurred (range 4 to 8 stimulations). The initial ADT in these rats was 152.1 ± 20.4 µA (range 75 to 240 µA); the first post-kindling ADT was 122.9 ± 14.3 µA (range 90 to 200 µA). The lowered post-kindling ADT reflect the progressively higher seizure susceptibility of the kindled compared to the non-kindled brain (Goddard et al., 1969). The first GST was 175.7 ± 25.5 µA (range 90 to 280 µA). During pre-drug control testing, all rats exhibited secondary generalized seizures at the ADT, so that ADT was identical to GST. The pre-drug control kindled seizure parameters were (mean ± SEM): ADT and GST, 57.2 ± 7.1 µA (range 30 to 135 µA); ADD, 93.4 ± 5.2 sec (range 66.5 to 136.5 sec); SD, 91.0 ± 5.3 sec (range 67.5 to 127.0 sec); LP, 6.4 ± 1.7 sec (range 1.0 to 26.5 sec). Seizure severity was classified as stage 5 according to Racine (1972) in all rats during pre-drug control testing. The pre-drug control kindled seizure parameters did not differ significantly between vehicle- and Sandimmune®-treated rats (p > 0.05 for all comparisons). Since this study was performed in a cross-over design, pre-drug control values were additionally compared before and after cross-over for each kindled seizure parameter, without indicating any significant differences (p > 0.05 for all comparisons).

3.4.5 Lack of Acute and Chronic Effects of CsA on Kindled Seizure Parameters

Just as for PTZ seizure thresholds, kindled seizure parameters were always determined 2 hrs after drug or vehicle injection. Sandimmune® 10 mg/kg i.p. did not cause significant changes in the kindled seizure parameters ADT, GST, ADD at ADT, ADD at GST, SD at ADT after acute or chronic drug injection, neither compared to pre-drug values nor compared to vehicle-treated rats (Fig. 5). Likewise, further investigated kindled seizure parameters (SD at GST, SS at ADT, SS at GST, LP) were not changed significantly by acute or chronic treatment with Sandimmune® 10 mg/kg i.p. (not illustrated).
Study 2: Effects of Cyclosporine A on Seizure Thresholds

3.4.6 Effects of CsA on Rat Behavior

To evaluate behavioral adverse effects of the different CsA treatment regimens after acute or chronic treatment, a set of behavioral tests was carried out starting 1 ¾ hrs after drug or vehicle injection. Additionally, behavioral tests were carried out on naïve rats. All behavioral tests and physiological measures were carried out on rats of the PTZ study. A general and qualitative overview of the results is given in Table 1. Variations in time of behavioral parameters significantly changed by specific CsA treatment regimens compared to pre-drug

Figure 5: Kindled seizure parameters over time shown as means and SEM relative to pre-drug control. Parameters were determined at different time points before (pre-drug), during (acute, 1 week (1 wk), 2 weeks (2 wk)), and after treatment (1 wk post). Rats were treated intraperitoneally with 10 mg/kg cyclosporine A as the ready-to-use preparation Sandimmune® (Sand, Novartis) or with vehicle. (A) Afterdischarge thresholds (ADT), (B) generalized seizure thresholds (GST), (C) afterdischarge durations at ADT, (D) afterdischarge durations at GST, and (E) seizure durations at ADT. Sample sizes are given within the bars. Treatment with Sandimmune® neither changed kindled seizure parameters compared to pre-drug values nor compared to vehicle treatment (p>0.05, all comparisons).
values and respective results from naïve rats are illustrated in Fig. 6. Most of the investigated behavioral parameters including excitability of rats were not affected by the different CsA treatment regimens (Table 1).

<table>
<thead>
<tr>
<th>Behavioral parameter</th>
<th>Results of the statistical analysis of the behavioral parameters for each treatment group</th>
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<td></td>
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<td>Hyperlocomotion</td>
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<td>Hypolocomotion</td>
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<td>Ataxia</td>
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<td>Tremor</td>
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<td>Head swaying</td>
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<td>Stereotypic snifing</td>
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<td>Straub tail</td>
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<td>Piloerection</td>
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<td>Retracted flanks</td>
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<td>Flat body posture</td>
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<td>Approach-response test</td>
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<td>Touch-response test</td>
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<td>Finger-snap test</td>
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<td>Pick-up test</td>
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<td>Abdominal muscle tone</td>
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<td>Number of feces boli</td>
<td>n.s.</td>
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<tr>
<td>Consistency of feces</td>
<td>n.s.</td>
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Table 1: Overview of behavioral and physiological (defecation) results for naïve rats and rats treated with different CsA preparations, dosages, vehicles, and application routes. Significant changes over time as analyzed by one-way ANOVA are indicated by the given p-values. Arrows indicate significant increases of specific behaviors or physiological measures during the course of the experiment as analyzed by posthoc Dunn’s multiple comparisons when compared to pre-drug control. Not significant test results are indicated by n.s. as abbreviation. Not significant posthoc test results are indicated by n.s. in brackets. For detailed illustration of significant parameters see Figure 6. n.s., one-way ANOVA not significant; [n.s.], posthoc test not significant.

Compared to pre-drug behavior, tremor was significantly induced in rats treated chronically for two weeks with pCsA 10 mg/kg i.p., Sandimmune® vehicle s.c., and Sandimmune® 10 mg/kg s.c. (Fig. 6B-D), while no such behavior was observed in naïve rats (Fig. 6A). The tremor was typically still detectable one week after discontinuation of treatment. Piloerection was significantly induced during chronic treatment with Sandimmune® 10
mg/kg s.c. (Fig. 6F), while this was never observed in naïve rats (Fig. 6E). After acute treatment with Sandimmune® 5 mg/kg i.p., 2 out of 8 rats retracted their flanks and this was statistically significant compared to the pre-drug time point (Fig. 6H). Although not significant, retracted flanks could also be observed in some naïve rats (Fig. 6G).

Figure 6: Behavioral and physiological (defecation) data shown as single values and median for (A-D) tremor, (E-F) piloerection, (G-H) retracted flanks, (I-L) number of feces boli, and (M-P) consistency of feces. Significant increases over time (compared to pre-drug control) after treatment with specific CsA preparations, dosages, vehicles, and application routes are indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001). Data from naïve rats are shown for comparison. For further information refer to Table 1.
3.4.7 Effects of CsA on Defecation

At the end of the behavioral tests, the number of feces boli in the open field and the consistency of feces were determined as further physiological measures. CsA in different preparations and also Sandimmune® vehicle (probably the ethanol contained therein) clearly affected the number of feces boli and/or the consistency of feces. After one or two weeks treatment, Sandimmune® vehicle i.p., Sandimmune® 10 mg/kg i.p., and Sandimmune® 5mg/kg s.c. significantly induced an increase in the number of feces boli when compared to pre-drug control (Fig. 6J-L). Except for Sandimmune® 10 mg/kg i.p.-treated rats, this increased number of feces boli was still significantly present up to two weeks after discontinuation of treatment. Naïve rats did not show significant changes over time for this measure (Fig. 6I). In rats receiving pCsA 10 mg/kg and both dosages of Sandimmune® i.p., soft feces or diarrhea was significantly induced after acute and during chronic treatment and returned to normality after discontinuation of treatment (Fig. 6N-P). Soft feces or diarrhea was observed only occasionally in naïve rats (Fig. 6M).

3.4.8 Effects of CsA on Body Temperature

The body temperature of the rats at the beginning of experiments (pre-drug control) was 37.94 ± 0.04°C (mean ± SEM; range, 37.1 to 39.1°C) with no differences between the treatment groups (one-way ANOVA, p = 0.09).

In general, both pCsA i.p. dosages, the respective vehicle, and Sandimmune® 10 mg/kg i.p. caused a significant decrease of relative body temperature during treatment (for more details refer to Fig. 3B). Sandimmune® 10 mg/kg i.p. caused an acute decrease in relative body temperature by about 2% (37.18 ± 0.21°C) after a single drug injection. After one week chronic treatment, the relative body temperature was still significantly lower than the pre-drug control. The pCsA preparations including vehicle caused a delayed significant decrease in relative body temperature during chronic treatment (Fig. 3B). Similarly, these treatment groups developed significantly decreased relative body temperatures compared to naïve rats or other groups during treatment. The relative body temperature of rats treated with Sandimmune® 10 mg/kg i.p. was significantly lower compared to naïve and other Sandimmune®-treated rats after acute drug injection. The relative body temperature of rats treated with pCsA or vehicle comparably decreased after acute treatment, but also during
chronic treatment. One and two weeks after discontinuation of treatment, relative body temperature has returned to pre-drug values, although some intergroup differences could be still detected two weeks post-treatment (Fig. 3B).

3.4.9 Effects of CsA on Body Weight

The body weight of rats was measured immediately before each PTZ seizure threshold determination. At the pre-drug control day, rats had a body weight of 228.6 ± 1.03 g (mean ± SEM; range, 211.2 to 269.5 g). Rats were then distributed randomly to the different treatment groups. The mean pre-drug control body weights did not differ significantly between the groups (one-way ANOVA, p = 0.49).

As expected, body weight significantly increased over time in all rats (Fig. 3C). Some treatment groups (pCsA 5 mg/kg i.p., pCsA 10 mg/kg i.p., pCsA vehicle i.p., Sandimmune® 10 mg/kg i.p., Sandimmune® 5 mg/kg s.c.) significantly gained weight already during treatment, as observed at the two week time point. One week post-treatment, all treatment groups significantly gained weight. Additionally, the body weight gain differed significantly between treatment groups (Fig. 3C). Naïve rats showed a body weight gained of 2.79 ± 0.55% at the 2 week-treatment point and 5.67 ± 0.94% at the 1 week-post-treatment time point. Compared to the naïve rats, many of the treated rats showed a significantly higher bodyweight gain: Rats treated with pCsA 10 mg/kg i.p. (6.87 ± 1.24%) at the 2 week-treatment time point and rats treated with pCsA 5 mg/kg i.p. (10.42 ± 1.33%), pCsA 10 mg/kg i.p. (9.73 ± 1.80%), Sandimmune® vehicle s.c. (11.71 ± 1.04%), Sandimmune® 5 mg/kg s.c. (9.86 ± 1.69%), and Sandimmune® 10 mg/kg i.p. (11.76 ± 1.10%) at the 1 week-post-treatment time point. Differences in body weight gain over time or between groups were not related to differences in the number of feces boli or the occurrence of diarrhea. For example, although rats treated with Sandimmune® 10 mg/kg i.p. showed a significant increase in number of feces boli (Fig. 6J) and significantly more rats showed soft feces or diarrhea (Fig. 6P) during treatment, they nevertheless significantly gained weight during the course of the experiment (Fig. 3C).
3.5 Discussion

The main findings of the present study were: (1) None of the investigated CsA preparations, dosages, and application routes caused robust seizure threshold changes in the timed intravenous PTZ seizure threshold test after both single CsA injection and chronic treatment. (2) Neither single injection nor chronic treatment of CsA in the ready-to-use preparation caused changes in amygdala-kindled seizure thresholds or other kindled seizure parameters. (3) Whole blood CsA levels accumulated over the first week of treatment and were higher after treatment with the ready-to-use preparation compared to pCsA and after i.p. compared to s.c. injection. (4) The occurrence of adverse effects (diarrhea, lowered body temperature, tremor, piloerection) differed between CsA regimens and some were also induced by vehicle injections containing ethanol and/or polyethoxylated castor oil. An increased occurrence of diarrhea was not accompanied by reduced body weight gain.

3.5.1 Lack of Robust CsA Effects on Seizure Thresholds

CsA is a neutral cyclic polypeptide with rather complex pharmacokinetics (Tanaka et al., 1999). It does not readily pass the blood-brain barrier due to a combination of binding by serum proteins and low permeability of the barrier related to efflux transport by the multidrug transporter P-glycoprotein (Cefalu and Pardridge, 1985, Lemaire et al., 1988, Sakata et al., 1994). As a result, significantly higher CsA levels can be measured in blood compared to brain (Boland et al., 1984, Bronster et al., 1999). Nevertheless, CsA is known to affect brain functions, partly because immunophilins are enriched far more in the brain than in the immune system (Steiner et al., 1992). Furthermore, CsA itself was reported to increase the permeability of the blood-brain barrier due to several mechanisms including direct impairment of endothelial cells (Kochi et al., 1999, Wijdicks, 2001, Dohgu et al., 2010). These mechanisms may be at least partly involved in the occurrence of CsA-induced encephalopathy. Furthermore, experimentally induced seizures including PTZ-induced seizures have been shown to cause blood-brain barrier impairment in several brain areas (e.g., Ziylan and Ates, 1989, Librizzi et al., 2012), thereby allowing larger amounts of CsA to penetrate into the brain. Noteworthy, the blood-brain barrier is adversely affected after cell grafting into the brain, thus allowing macromolecules and leukocytes to pass for about 1 to 2
weeks (Brundin et al., 1989). Thus, treatment with immunosuppressive drugs such as CsA requires particular caution in neural transplantation studies, especially in epilepsy research. However, apart from a significant induction of tremor in some rats, we did not observe any obvious neurological adverse effects after acute or during chronic treatment with CsA and also did not observe robust effects on seizure thresholds. This might be partly related to an increased expression of P-glycoprotein in response to repeated CsA exposure, as was shown in human arterial endothelial cell culture experiments (Hauser et al., 1998). We did not determine CsA levels in brain tissue in the present study, but it is interesting to note that a study by Gottschalk et al. (2011) indicated that female rats reach lower CsA levels within the brain and are less susceptible to neurotoxic or neurobehavioral adverse effects by CsA than male rats. This may also explain why Borlongan et al. (1995, 1999) described that CsA injected at therapeutic doses caused spontaneous hyperactivity in normal male rats, whereas we did not observe such effects on motor behavior in female rats. Our study, in which comparable dosages were used, rather indicated slight hypolocomotion. This discrepancy may additionally be explained by different CsA preparations used in their studies and in our investigation.

A considerable amount of previous in vivo and in vitro studies investigated putative CsA effects on different seizure parameters. In vitro studies using rodent brain slice recordings either showed an increase (Wong and Yamada, 2000) or a decrease (Jung et al., 2012) of epileptiform activity by CsA, which may depend on the basal seizure susceptibility of a neuronal network rather than on the used CsA concentration (Gorji et al., 2002). Likewise, a broad range of proconvulsant (Racusen et al., 1988, Famiglio et al., 1989, Racusen et al., 1990, Asanuma et al., 1995b, Shuto et al., 1999, Fujisaki et al., 2002, Setkowicz et al., 2004, Yamauchi et al., 2005) or anticonvulsant effects (Moia et al., 1994, Asanuma et al., 1995a, Homayoun et al., 2002, Setkowicz and Ciarach, 2007, Jung et al., 2012) were also reported in previous in vivo studies using rats and mice. Again, it seems that the used dosages do not predict the outcome of the investigations. This is in line with clinical findings emphasizing that there is no absolute correlation between serum CsA levels and seizure occurrence (Schwartz et al., 1995, Hauben, 1996). The different study outcomes are more likely related to the wide variability in general study design used by the different investigators. Dosages used in these studies ranged from 5 to 50 mg/kg, application routes included i.p., s.c., and
oral, and several different acute or chronic treatment schedules were applied. Furthermore, different CsA preparations were used and in most cases the CsA sources were not even specified.

Finally, CsA effects on seizure parameters were evaluated using varying seizure or epilepsy models in rats and mice of different strains and gender. None of these experimental factors alone seem to determine, if CsA exerts pro- or anticonvulsant effects, but rather a combination of several parameters shifts the outcome in one or the other direction. The present study therefore comprehensively and directly compared different treatment regimens regarding CsA preparations, dosages, application routes, treatment duration, and experimental rat models. However, none of the used treatment regimens caused robust changes in PTZ or kindled seizure thresholds and further seizure parameters, although the severity and amount of adverse effects differed depending on the treatment paradigm.

There have been two previous studies on CsA effects in rodents using the same seizure and epilepsy models as we did in our investigation, albeit male mice and rats instead of female rats were used. Using male mice, Homayoun et al. (2002) observed an elevation of the clonic seizure threshold in the i.v. PTZ model after a single s.c. injection of CsA in the ready-to-use preparation. In that study, saline was used as vehicle-control, although the ready-to-use preparation contains ethanol, known to be anticonvulsant per se, albeit not in the concentration used after dilution of the ready-to-use preparation, as indicated by our study. Nevertheless, in our study in female rats, we observed a similar elevation of the clonic seizure threshold, but only after chronic treatment with a rather low dosage (5 mg/kg) of CsA applied i.p. as the ready-to-use preparation. Using male rats, Moia et al. (1994) reported that the kindling progression from partial to secondarily generalized seizures induced by electrical stimulation of the left amygdala was reversibly blocked by 10 mg/kg CsA in the ready-to-use preparation applied i.p., indicating an anticonvulsant effect. In our study in rats fully kindled by electrical stimulation of the right amygdala, we did not observe any indication for an anticonvulsant effect by CsA using the same dosage, application route, and preparation. This discrepancy might be related to the different rat strains (Sprague Dawley and Wistar, respectively) and/or gender used by Moia et al. (1994) and in our study. It is interesting to note that Tauboll et al. (1998) also did not observe robust CsA effects on recordings in naïve hippocampal slices prepared from female rats. Species-, strain-, and
gender-specific effects thus cannot be ruled out, although Santos and Schauwecker (2003) also have shown a similar lack of CsA effects on the induction of kainate-induced seizures in male mice. Our investigation together with previous reports suggest that transplantation studies in experimental epilepsy research require a careful assessment of putative CsA effects on seizure thresholds or further seizure parameters in the specific experimental settings to be used.

3.5.2 Bioavailability of CsA

It is well-known that the bioavailability (Luke et al., 1990) and pharmacokinetic profiles (Wassef et al., 1985) are influenced by the route of administration of CsA in rats. Accordingly, it was recently shown that the survival of human neural cells grafted into the male rat brain differed depending on the administration route of CsA applied chronically for about 2 weeks with better outcome after s.c. injection compared to sole oral administration (Jensen et al., 2012). Our data suggest that for a chronic pharmacological immunosuppression with CsA in the PTZ seizure threshold and amygdala-kindling model in rats, the ready-to-use preparation in a dose of 10 mg/kg applied i.p. is the most appropriate and safe treatment regimen. This treatment protocol induced the highest whole blood CsA levels, thus showing the most effective absorption, but nevertheless did not affect seizure thresholds in the two rat models and did not cause stronger adverse effects than the other treatment regimens investigated. In line with our observation that whole blood CsA levels accumulated over the first week of treatment, Boland et al. (1984) showed that repeated administration of CsA after the first week did not further elevate CsA tissue concentrations in mice. Our treatment regimens involving i.p. administration of CsA thus yielded whole blood levels within the range of previously described therapeutic CsA blood concentrations in rats (Chimalakonda et al., 2002, Brocks et al., 2006). In contrast, when we applied CsA via s.c. route, whole blood levels were much lower, which may add to the complete lack of effects of s.c. applied CsA on seizure thresholds.

3.5.3 Adverse Effects Induced by CsA

We could show that CsA is relatively safe for use in the PTZ seizure threshold test and the kindling model in rats. It nevertheless caused different adverse effects, which mainly
comprised diarrhea, a decreased body temperature, and tremor. Although a relatively common adverse effect in humans, we did not evaluate putative nephrotoxic adverse effects of CsA in our study, because rats appear to be rather unsusceptible to nephrotoxic CsA effects (Morozumi et al., 1986).

In line with our observation in rats, diarrhea is a known adverse effect of CsA treatment in humans (overview by Ginsburg and Thuluvath, 2005). With respect to our observed drop in body temperature, CsA has been reported before to interfere with thermoregulation and to cause hypothermia in rats (Dantzer et al., 1987). However, we also observed a drop in body temperature in response to vehicle injections containing polyethoxylated castor oil.

Several mechanisms of action including reduced GABAergic inhibition, mitochondrial dysfunctions, and cerebral vasculopathy have been suggested to be involved in CsA neurotoxicity, which may express as acute encephalopathy, tremor, and CsA-induced seizures (Hauben, 1996, Gijtenbeek et al., 1999, Bechstein, 2000, Serkova et al., 2004). In our study, none of the investigated rats showed clinical signs of spontaneous seizures. Instead, we observed tremor in a significant portion of rats after two weeks chronic treatment and the tremor persisted for about one week after discontinuation of treatment. Tremor is among the most commonly reported neurological adverse effects in humans (Gijtenbeek et al., 1999, Bechstein, 2000). However, in our rats tremor was induced not only by higher doses of CsA preparations, but also by repeated injections of vehicle containing Cremophor® EL and ethanol. In this regard, it is interesting to note that a previous in vitro study by Windebank et al. (1994) also indicated neurotoxic effects of the solvent vehicle for CsA, because polyethoxylated castor oil itself induced neurotoxic effects including axonal swelling, degeneration and demyelination even at low doses.

3.6 Conclusion

Chronic daily treatment with CsA in a ready-to-use preparation injected i.p. for up to two weeks is potentially safe for preclinical neuronal xenotransplantation studies in epilepsy research. In the intravenous PTZ seizure threshold model and in the amygdala-kindling model for temporal lobe epilepsy in rats, no robust influences on seizure thresholds and further seizure parameters were detected. Nevertheless, a careful monitoring of rats chronically treated with any preparation of CsA should be ensured because of the risk for
adverse effects, which may occur during chronic treatment. Indeed, the immunosuppression protocol elaborated in the present study should be further developed for an application in future xenotransplantation studies in experimental epilepsy research. For example, although some studies suggest that a transient CsA treatment for about 2 to 3 weeks might be sufficient for a longer survival time of human neural progenitor cells in rats (Wennersten et al., 2006, Rota Nodari et al., 2010), other studies reported xenograft rejections after cessation of CsA treatment in rats (Brundin et al., 1989, Honey and Shen, 1999). We did not investigate CsA effects beyond 2 weeks of chronic treatment, although neural progenitor cells typically need much more time to fully differentiate and integrate into the host rat brain. We cannot exclude potential effects on seizure thresholds in response to longer CsA treatment durations. Furthermore, long-term treatment with CsA is associated with higher risks of adverse effects. Thus, additional research beyond the scope of the present study may help to find an optimized protocol. Such a protocol should avoid the observed adverse effects of CsA and at the same time should reliably ensure long-term xenograft survival, because Larsson et al. (2001) indicated that 10 mg/kg CsA injected i.p. once daily failed to protect porcine neural xenografts from rejection after treatment durations extending beyond 12 weeks. Jensen et al. (2012) suggested a protocol, which avoids the use of frequent (daily) injections by applying a combination with oral treatment subsequent to initial post-surgery injections. Kelly et al. (2009) successfully reported neonatal induction of tolerance to the cells to be grafted in adulthood as an alternative to pharmacological immunosuppression.

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CONFLICT OF INTEREST: None.
3.7 References


STUDY 2: EFFECTS OF CYCLOSPORINE A ON SEIZURE_THRESHOLDS


STUDY 2: EFFECTS OF CYCLOSPORINE A ON SEIZURE THRESHOLDS


4 IMMUNOMODULATION FOR LONG-LASTING ANTICONVULSANT EFFECTS AFTER NEURAL XENOGRRAFTING IN RATS – NEONATAL INDUCTION OF TOLERANCE VERSUS PHARMACOLOGICAL IMMUNOSUPPRESSION

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4.1 Abstract

Neurotransplantation is one promising experimental approach to overcome the problem of pharmacoresistance in epilepsy. Depending on the grafted cell type (e.g. xenotransplantation), an immunosuppression is necessary to prevent tissue rejection. Although occasionally associated with severe adverse effects, a pharmacological immunosuppression with cyclosporine A (CsA) is commonly used for xenotransplantations in experimental animal models. An alternative immunomodulatory approach is the neonatal induction of tolerance towards the cells intended for later transplantation. In the present study, porcine neural precursor cells (pNPCs) were comparatively grafted into the subthalamic nucleus (STN) of rats, which were either (1) pharmacologically immunosuppressed with CsA, (2) neonatally tolerance-induced, or (3) not immunomodulated at all. Bilateral grafting of pNPCs into the STN, known to be involved in seizure modulation, was significantly anticonvulsant in all animal groups. However, only animals that were neonatally tolerance-induced showed longer-lasting significant anticonvulsant effects until 5 weeks after grafting, whereas these effects only lasted 10 days in the other groups. Compared to pharmacological immunosuppression with CsA, the induction of tolerance did not cause any obvious adverse effects. Although the observed long-lasting anticonvulsant effects in tolerance-induced rats were still transient, our promising data on neonatal induction of tolerance and the use of pNPCs for xenotransplantation studies in epilepsy research merit further investigations.

Key words: Neurotransplantation; Porcine precursor cells; Basal ganglia; Epilepsy; Cyclosporine A; Desensitization; Pentylenetetrazole

4.2 Introduction

Neural transplantation into appropriate brain regions is a promising experimental approach to treat pharmacoresistant epilepsies. The subthalamic nucleus (STN), a basal ganglia structure known to be involved in the modulation of seizures emanating from the limbic system (Depaulis et al., 1994; Gale et al., 2008; Kücker et al., 2010), has recently been shown to be a highly promising target region for localized treatment approaches including intracerebral drug injections and neuronal transplantations (Bröer et al., 2012; Gernert,
2013; Handreck et al., 2014). Using cell lines that release the inhibitory neurotransmitter γ-aminobutyric acid (GABA), Handreck et al. (2014) for the first time demonstrated anticonvulsant effects by allografting rat cells into the rat STN.

However, for clinical translation of a cell-based therapy in epilepsies, other cell sources such as neurally directed human or porcine cells have to be investigated preclinically to demonstrate safety and efficacy after grafting into the brain (Schachter et al., 1998; Brevig et al., 2000). Especially porcine tissue raises less ethical concerns than the use of human fetal tissue. Because xenografts are rejected rapidly in rats and mice, an immunosuppression to prevent graft rejection and to promote long-term anticonvulsant effects is inevitable (Pakzaban and Isacson, 1994; Brevig et al., 2000; Cascalho and Platt, 2001). The immunosuppressive drug cyclosporine A (CsA) (Kahan, 1989, 2009) is commonly used for this purpose. We recently reported that the chronic treatment with intraperitoneally (i.p.) applied CsA (10 mg/kg) from a ready-to-use preparation is an appropriate immunosuppressive regimen, because it causes high CsA whole blood concentrations without robustly affecting seizure thresholds in an acute seizure model (Handreck et al., submitted). However, typical adverse effects such as gastrointestinal problems were caused by the treatment. Furthermore, CsA treatment periods larger than two weeks were not investigated by Handreck et al. (submitted), but may be associated with higher risks for severe adverse effects. Finally, repeated drug injections for longer times can be considered to be much more stressful for the animals.

An alternative strategy to promote long-term graft survival, without the need of repeated drug injections, is the neonatal induction of tolerance towards the cells intended for later transplantation. This method has been employed for decades with various types of tissue (e.g. liver cells, bone marrow cells, spleen cells) in different transplantation approaches (Billingham et al., 1953; Billingham and Brent, 1956; Ando et al., 1991; West et al., 1994a, b; Ridge et al., 1996; Modigliani et al., 1997; Adkins et al., 2004; Peiguo et al., 2012). More recently, neonatal induction of tolerance was shown for the first time to successfully allow long-term survival of neural tissue grafts (Kelly et al., 2009). However, there are also studies reporting a failure of neonatal tolerance induction for neural transplantation, suggesting that the efficacy of this immunomodulatory strategy depends on the host species, the tissue used for grafting, and maybe the target region within the host brain (Janowski et al., 2012;
Roberton et al., 2013). The grafting of clinically relevant porcine donor cells has not yet been tested in tolerance-induced rats.

In the present study, we therefore investigated the efficacy of a transplantation of porcine neuronal precursor cells (pNPCs) into the STN in an acute seizure model. To directly compare the tolerance induction as an immunomodulatory strategy with a conventional pharmacological immunosuppression regarding feasibility, safety, and ability to promote long-term graft survival, we transplanted the cells into rats, which were either neonatally tolerance-induced, treated with CsA, or not immunomodulated at all. Adverse effects were comparatively investigated using a battery of behavioral and physiological tests.

4.3 Material and Methods

An overview of the study design is illustrated in Figure 1.

Figure 1: Time line illustrating procedures conducted during the present study. Porcine neuronal progenitor cells (pNPC) were grafted into the subthalamic nucleus (STN) of rats with different immunomodulations: without immunomodulation (Graft), immunosuppression with cyclosporine A (CsA+Graft), or induced tolerance (Tol+Graft). As control, rats received CsA without cell transplantation. Behavioral tests and pentylenetetrazole (PTZ) seizure threshold determinations were conducted repeatedly at different time points. Refer to the text for details. i.p., intraperitoneally; P, postnatal day.
4.3.1 Animals

For rat breeding, adult male and female Wistar rats (Harlan-Laboratories, Horst, Netherlands) were paired by housing together for two days. Pregnant rats then were housed individually. For comparison with previous grafting studies of our group (Löscher et al., 1998; Gernert et al., 2002; Nolte et al., 2008; Gernert et al., 2011; Handreck et al., 2014), we only used female offspring. Three weeks after birth, female pups (n = 72; including n = 22 with induced immunological tolerance as described below) were weaned and housed in groups of four to five in an separate animal husbandry facility without males in order to keep them asynchronous with respect to their estrous cycle (Kücker et al., 2010). Experiments were started when rats reached a body weight of 200-220 g. All rats were kept under controlled environmental conditions with a 12-h/12-h light/dark cycle (light on at 6:00 a.m.). Standard laboratory chow (Altromin 1324 standard diet) and tap water were allowed ad libitum. All animal experiments were done in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and were formally approved by the animal subjects review board of our institution. All efforts were made to reduce the number of animals used and their suffering. For transplantation, porcine cells were prepared from pig fetuses. Parental pigs were housed and handled according to the German guidelines for animal welfare. The animal experiments were approved by an external ethics committee (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, AZ 33.9-42502-04-09-1718).

4.3.2 Cell Cultivation

Unless otherwise stated, all reagents were purchased from PAA Laboratories (Marburg, Germany). Knockout DMEM F12, Neurobasal®, StemPro®, Fungizone® and Accutase® were purchased from Life Technologies™ GmbH (Darmstadt, Germany). Fibroblast growth factor (FGF-2, 20 ng/ml) and epidermal growth factor (EGF, 20 ng/ml) were obtained from PAN-Biotech™ GmbH (Aidenbach, Germany). DNase I (0.05%) was purchased from Roche AG (Berlin, Germany). Cell culture flasks were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany).
**4.3.2.1 Preparation of Primary Cell Culture**

The protocol for isolation of primary porcine mesencephalic cells was adapted from Armstrong et al. (2003). In brief, fetuses at embryonic day E25 were derived by sterile hysterectomy from pregnant sows (German Landrace) after head-only electrical stunning and bleeding. The ventral mesencephali (VM) were dissected from an entire litter and prepared as a single cell suspension with Accutase®, DNase, and mechanical trituration by using fire polished pasteur pipettes. Cells of each fetus were seeded in cultivation medium composed of knockout DMEM F12 supplemented with 2% StemPro®, penicillin/streptomycin/Fungizone® (PSF, 100 mg/ml), EGF (20 ng/ml), and FGF (20 ng/ml). Precursors were grown at 37°C (95% humidity, 3% CO₂) and fed every third day by replacing half the medium with fresh medium. After 7-10 days, the pNPCs were retrieved by scraping lightly attached spheres from the culture flask and subsequent centrifugation to remove the supernatant. Spheres were incubated in Accutase® for 5 min at room temperature and were then carefully dissociated with a fire polished Pasteur pipette. Cell number and viability were determined by using the trypan blue exclusion test. Cells were reseeded at a density of 200,000 cells/ml in cultivation medium, maintained for 7 days as described above, and prepared for induction of tolerance as described below.

**4.3.2.2 Preparation of pNPCs for Tolerance Induction and Neurotransplantation**

The pNPC spheres were dissociated to single cells via Accutase® digest and mechanical trituration and counted with the help of a haemocytometer using the trypan blue exclusion test. Shortly before injection of the cells, precursors were resuspended in grafting medium (100,000 cells/µl) (Neurobasal®). Remaining cells were kept in cultivation medium for one week, centrifuged to remove supernatant, and resuspended in freezing medium composed of cultivation medium, 10% DMSO (Sigma-Aldrich GmbH, Steinheim, Germany), and 20% StemPro®. pNPCs were kept at -20°C for 30 minutes and stored at -80°C over night. After 16h hours vials were transferred to fluid nitrogen. One week before transplantation, pNPCs were thawed and cultivated under standard conditions. At the day of transplantation, cells were dissociated to single cells via Accutase® digest and mechanical trituration and counted under a haemocytometer again using the trypan blue exclusion test. Cells were resuspended in Neurobasal medium (80,000 pNPCs/800 nl) and grafted immediately.
4.3.3 Induction of Immunological Tolerance

For induction of immunological tolerance, two female pups per litter were randomly selected at postnatal day 1-2 and briefly separated from the mother. A suspension of 100,000 pNPCs in one µl DMEM/F12 medium was injected i.p. using a handheld 5 µl Hamilton glass microsyringe with removable 26 gauge needles (Sigma-Aldrich GmbH, Steinheim, Germany) (Kelly et al., 2009). For correct identification of rats with induced tolerance during the entire study period, the bottom of the right hind paw of the pups was tattooed with green animal tattoo ink (Fine Scientific Tools GmbH, Heidelberg, Germany) using a 26 gauge needle. Pups were handled wearing nitrile gloves and were immediately returned to the litter in order to avoid rejection by the mother. Due to death of 4 animals, 22 from initially 26 pups receiving i.p. injection of pNPCs could be weaned and used in the present study. They were housed together with rats, in which no immunological tolerance was induced to ensure identical handling of all animals.

4.3.4 Pharmacological Immunosuppression

For pharmacological immuno-suppression with CsA, rats were treated with a dilution (1:15) of the ready-to-use drug Sandimmune® (50 mg/ml; Novartis Pharmaceuticals). Rats received 10 mg/kg CsA i.p. daily (injection volume, 3 ml/kg) over a period of 15 days. In animals transplanted with pNPCs, immunosuppressive treatment was started one day before surgery. As shown previously by our group (Handreck et al., submitted), an immunosuppression with 10 mg/kg CsA injected i.p. using Sandimmune® had no robust effects on acute seizure thresholds in the test, which was also used in the present study. Nevertheless, we added a CsA-treated control group without transplantation to confirm our previous results. Therefore, some animals received an immunosuppression with 10 mg/kg CsA i.p. (15 days) without being transplanted (Fig. 1).

4.3.5 PTZ Seizure Threshold Test

For investigating seizure thresholds before and at different times after transplantation of pNPCs into the STN, the timed intravenous pentylenetetrazole (PTZ, metrazol) seizure threshold test was performed as described in detail previously (Redgrave et al., 1992; Löscher, 2009; Bröer et al., 2012). This test can be repeatedly performed in the same rat at
intervals of about 48 h, thus allowing the study of alterations in seizure thresholds in individual rats (Löscher, 2009). To prevent a kindling effect, in the present study the number of PTZ seizure threshold determinations was limited to a maximum of five (with intervals of at least ten days) in the same rat.

The pre-drug control seizure threshold was determined two to three days before cell transplantation. Thus, each rat served as its own control. Further PTZ seizure thresholds were then determined 10/11 days, 3 weeks, 5 weeks, and 3 months after grafting of pNPCs.

In groups receiving an immunosuppression by CsA, PTZ seizure thresholds were determined 19–21 hours after injection of CsA, i.e. at about trough levels of CsA within the blood. Seizure threshold determination was always performed at the same time of the day (between 9:00 and 12:00 a.m.) to avoid intraday variance between animals. The experimenter was blinded with respect to the group affiliation of the rats.

PTZ seizure thresholds were determined by infusion of a 0.8% solution of PTZ (Caesar & Loretz, Hilden, Germany) in saline via a 24-gauge needle (Terumo® Europe n.V., Leuven, Belgium) into the lateral tail vein of the conscious, unrestricted rat. The PTZ solution was infused through a flexible polyethylene tubing (Kleinfeld Labortechnik, Gehrden, Germany) using an infusion pump (PHD 2000 Infusion, Harvard Apparatus, Holliston, MA, USA). Immediately after occurrence of the first clonic seizure, the infusion was terminated. The PTZ seizure threshold was calculated in mg PTZ per kg body weight based on the time needed to induce this clonus, the body weight of the animal, the rate of PTZ infusion (1.0 ml/min), and the PTZ concentration. Body weights were recorded shortly before PTZ seizure threshold determinations. Additionally, the threshold for induction of the first myoclonic twitch, which always preceded the clonic seizure, was calculated. All further seizures or other abnormal behavior occurring after the termination of PTZ infusion were noted. Therefore, rats were closely observed until they resumed normal behavior, typically about 1–2 h after PTZ infusion.

From initially 72 animals, 6 rats died after determination of the pre-drug control PTZ seizure threshold. The remaining rats, if not tolerance-induced, were randomly assigned to the different other study groups. Group sample sizes then were: pharmacological immunosuppression with 10 mg/kg CsA i.p. but no transplantation (CsA), n = 6; pNPC transplantation without immunomodulation (Graft), n = 20; pNPC transplantation and
immunosuppression with 10 mg/kg CsA i.p. (CsA+Graft), n = 20; pNPC transplantation after neonatal induction of tolerance (Tol+Graft), n = 20 (Fig. 1). Because some animals died after PTZ infusion and because of repeated paravenous PTZ infusion in some animals, not all time-points of seizure threshold determinations were available for analyses.

4.3.6 Transplantation of Porcine Neuronal Precursor Cells

For cell grafting, animals were anesthetized with isoflurane (induction 3%, maintenance 1.5%; CP-Pharma, Burgdorf, Germany) via an inhalation mask adjusted to a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Animals were mounted to the stereotaxic device, received additional local anesthesia of the scalp (2% tetracaine hydrochloride, Caesar & Lorez) and the periost (0.25% bupivacaine hydrochloride; Carbostesin®, AstraZeneca, Wedel, Germany), and were prepared for transplantation. Deposits of 80,000 cells in 800 nl were bilaterally grafted (one deposit per hemisphere) to the STN using the following stereotaxic coordinates in millimeters relative to bregma according to Paxinos and Watson (2007): posterior, –3.6; lateral, ±2.6; and ventral, –7.9 mm (Handreck et al., 2014). Cells were implanted using a 5 µl Hamilton syringe (outer tip diameter, 570 µm; inner tip diameter, 310 µm; Reno, NV, USA). The Hamilton syringe was inserted, left in place for 2.5 min before 400 nl of cell suspension were infused over a period of 30 s, left in place for another 2.5 min before the remaining 400 nl were infused over 30 s. The Hamilton syringe was again left in place for 2.5 min after the end of injection and controlled for permeability after removal from the brain. After suturing (Johnson-Johnson Intl., New Brunswick, NJ, USA) the scalp, isoflurane anesthesia was terminated and animals were closely observed for any behavioral alterations after recovering from anesthesia.

4.3.7 Behavioral Tests

To evaluate, if intrasubthalamic grafting of pNPCs alone or grafting in combination with CsA treatment or tolerance induction changes the behavior of the host animals, grafted rats were observed in the open field (OF) and the elevated plus maze (EPM) test 3 to 4 days before and 2 weeks (13 days; last day of immunosuppression with CsA) after transplantation (Fig. 1). Behavioral tests were done in a blinded manner with respect to the treatment of the animals. Tests were always performed at the same time of the day (behavioral observations
in the OF between 9:00 and 12:00 a.m., EPM test between 14:00 and 16:00 p.m.) to avoid intraday variance between animals.

4.3.7.1 Open Field

Behavioral observations in the OF were carried out 20-23 hours after CsA administration in the group receiving the pharmacological immunosuppression. Therefore, rats of all groups were transferred in their home cages from the animal facility to a lab provided for this purpose. After acclimatization, rats were placed individually in the center of a round, black-colored polyethylene open field (80 cm diameter, 25 cm height, 0.4 cm wall thickness) and observed for two minutes without disturbance. The following behavioral parameters were then rated using scores adapted from Hönack and Löscher (1995) as described recently (Handreck et al., submitted): Hypolocomotion (0, not observable; 1, tendency towards decreased locomotion; 2, clearly decreased locomotion with many breaks; 3, no forward movement), hyperlocomotion (0, not observable; 1, tendency towards increased locomotion; 2, clearly increased locomotion; 3, highly increased locomotion and hectic movements without any breaks), and ataxia (0, not observable; 1, slight ataxia with weak stagger; 2, clear ataxia with loss of balance during forward movement; 3, permanent loss of righting reflex despite the attempt to move forward). Tremor, head swaying, circling, stereotyped sniffing, straub tail, piloerection, and flat body posture were additionally scored (0, not observable; 1, suspicious; 2, observable; 3 intense).

Subsequently, the hyperexcitability test was carried out to detect differences in behavioral excitability and sensory responsiveness between the different study groups (Moser et al., 1988). In the approach-response test, a pen is moved slowly and vertically towards the nose of the rat and the response was scored as 1, no reaction; 2, sniffing at the pen; 3, moving away from the pen; 4, freezing; 5, jumping away from the pen; 6, attacking the pen. In the touch-response test, the rat is gently touched at the rump with the blunt end of a pen (response score: 1, no reaction; 2, turning slowly towards the pen; 3, moving away from the pen; 4, freezing; 5, jerking around towards the touch; 6, turning away from the touch; 7, jumping with or without vocalization). In the finger snap test, a standardized click noise is triggered above the back of the rat (response score: 1, no reaction; 2, normal reaction (moderate jumping, freezing, flinching, or flicking the ear); 3, intense jumping). In the pick-
up test, the rat is grasped around the body and lifted up slightly (response score: 1, very easy; 2, easy with vocalizations; 3, rearing and turning towards the hand; 4, avoiding the hand and trying to escape; 5, intense jumping; 6, attacking the hand). Additionally, the abdominal muscle tone was tested by palpating the abdomen of the rat (0, decreased; 1, normal; 2, increased), the number of solid feces boli was counted, and the consistency of the feces was rated (0, solid; 1, soft; 2, diarrhea). Finally, the rectal body temperature and the body weight of the rats were measured.

4.3.7.2 Elevated Plus Maze Test

Starting 1.5 hours after administration of CsA in the group receiving the pharmacological immunosuppression, rats of all groups were transferred in their home cages to a behavioral lab, where the EPM test was carried out. The EPM is an established test for assessing anxiety-like behavior in rodents (File and Day, 1972). Additionally, we used this test to quantify and evaluate the locomotion of the rats more precisely than this has been done previously by observing and scoring them in the open field (Handreck et al., submitted). The EPM was made of black polyethylene plastic and consisted of two open arms (50 cm × 14 cm), two enclosed arms (50 cm × 14 cm × 30 cm; wall thickness 0.9 cm), and a central platform (14 cm ×14 cm). The apparatus was elevated 86 cm above floor level and was illuminated with 77—94 lx at the surface of the open arms and 13 lx in the closed arms. Before each trial, the EPM was cleaned thoroughly with 0.1% acetic acid solution.

At the beginning of the trial, each rat was placed on the central platform always facing the same open arm. Each trial lasted 5 minutes. The behavior of rats was analyzed using the EthoVision 7.0 Software (Noldus Information Technology, Netherlands). The following parameters were measured: total distance moved (m) and velocity of movement (m/s) to measure horizontal locomotor activity, time spent in the different sections (open arms, closed arms, center), number of entries into every section, and latency time until first entry into a closed or open arm to measure anxiety-like behavior. Additionally, we manually investigated the number and duration of head dips over the edge of the open arms as well as the number and duration of rearing in every section to measure vertical locomotor activity.
4.3.8 **Histological Verification of Graft Localization**

At the end of the study period, i.e. 3 months after grafting, all rats were deeply anesthetized with chloral hydrate (AppliChem GmbH, Darmstadt, Germany) and transcardially perfused with 100–150 ml of 0.01 M PBS (pH 7.6) followed by about 240 ml of 4% paraformaldehyde in 0.2 M phosphate buffer (PB) (pH 7.6). The brains were removed and cryoprotected in 10% sucrose in 0.1 M PB (pH 7.6), followed by 30% sucrose 1–3 days later, and stored at 4°C. Three series of coronal sections were cut at 40 µm on a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany). One series was Nissl stained with thionine (Sigma-Aldrich) for verification of transplantation sites. The remaining two series were frozen at -20°C and stored for upcoming detailed immunohistochemical analysis and measurement of graft volume.

4.3.9 **Statistical Analysis**

Statistical analysis of data was performed depending on whether data were normally distributed or not. Data of PTZ seizure thresholds, EPM parameters, body weight, and body temperature were normally distributed, as verified with the Kolmogorov-Smirnov and the D’Agostino and Pearson tests. To detect putative intergroup differences at each time-point, data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests. To detect changes of PTZ seizure thresholds over time within each group (time-points after cell transplantation were compared to control threshold), one-way ANOVA analysis was used followed by Dunnett’s test. Because of missing data due to death of animals or paravenous PTZ infusion, we did not use repeated measurement analyses of seizure thresholds. Putative differences of EPM parameters, body weight, and body temperature between the two investigated time-points were analyzed by using paired t-tests.

Non-parametric data of behavioral scores were analyzed using Kruskal–Wallis ANOVA followed by Dunn’s multiple comparison test to detect intergroup differences. Changes between the time-points within each group were analyzed using the Wilcoxon signed rank test. All statistical analyses were performed with the Prism 5.03 software from GraphPad (La Jolla, CA, USA). All tests were used two-tailed and a value of $p \leq 0.05$ was considered significant.
4.4 Results

4.4.1 Localization of Grafted pNPCs

Localization of transplants was analyzed 3 months after implantation in individual rats (Fig. 1). Transplants were localized bilaterally within the STN (Graft group, n = 13; CsA+Graft group, n = 13; Tol+Graft group, n = 14), unilaterally within the STN (Graft group, n = 3; CsA+Graft group, n = 4; Tol+Graft group, n = 3), or bilaterally outside the STN (Graft group, n = 3; CsA+Graft group, n = 2; Tol+Graft group, n = 1). Some animals could not be included in final analysis due to different reasons (Graft group, n = 1 repeated paravenous PTZ infusion; CsA+Graft group, n = 1 graft only detectable in one hemisphere; Tol+Graft group, n = 2 false density of transplanted cell suspension).

In rats, in which the grafts were placed unilaterally outside the STN, the transplants were found dorsal to the STN within the zona incerta (n = 1 for Graft group, n = 3 for CsA+Graft group), lateral to the STN (n = 1 for Graft group), or anterior to the STN (n = 1 for Graft group, n = 1 for CsA+Graft group, n = 3 for Tol+Graft group). In cases in which the grafts were bilaterally placed outside the STN, the transplants were found dorsal to the STN within the ventral posteromedial nucleus (n = 1 for Graft group), anterior to the STN (n = 2 for Graft group, n = 1 for CsA+Graft group, n = 1 for Tol+Graft group), or at different locations on both sides (n = 1 lateral to the STN left and zona incerta right for CsA+Graft group).

4.4.2 Basal (control) PTZ Seizure Thresholds

The PTZ seizure thresholds before transplantation (control) did not differ (p = 0.095 for myoclonic twitch, p = 0.087 for clonic twitch) between tolerance-induced rats and rats of the three other study groups (CsA, Graft, Graft+CsA) after determination of the control seizure threshold. PTZ seizure thresholds for induction of the first myoclonic twitch before transplantation were 19.59 ± 0.6 mg/kg for the Graft group, 20.09 ± 0.62 mg/kg for the CsA+Graft group, 19.86 ± 0.66 mg/kg for Tol+Graft group, and 19.73 ± 0.78 mg/kg for CsA group. PTZ seizure thresholds for induction of clonic seizures before transplantation were 22.65 ± 0.71 mg/kg for the Graft group, 23.25 ± 0.8 mg/kg for the CsA+Graft group, 22.81 ± 0.61 mg/kg for the Tol+Graft group, and 23.57 ± 0.51 mg/kg for the CsA group.
4.4.3 Anticonvulsant Effects of pNPC Transplantation in the PTZ Seizure Threshold Test

In line with recent preliminary findings (Backofen-Wehrhahn et al., manuscript in preparation), bilateral transplantation of pNPCs into the STN caused a significant increase of mean seizure thresholds for induction of the clonic seizure 10/11 days after grafting compared to the control value in all grafted groups (Fig. 2B). Compared to control clonic seizure thresholds, the seizure thresholds were raised to 128 ± 4.9% in the Graft group (p < 0.001), 123.8 ± 7.1% in the CsA+Graft group (p < 0.01), and to 125.2 ± 3.4% in the Tol+Graft group (p < 0.001). Furthermore, the clonic seizure thresholds of these groups were significantly higher compared to the mean clonic seizure threshold of the CsA group 10/11 days after grafting (p < 0.01 for Graft group, p<0.05 for Graft+CsA and for Graft+Tol group). Animals of the CsA group showed stable seizure thresholds over the entire study period. Only rats of the Tol+Graft group showed an additional delayed increase of clonic seizure thresholds (116.3 ± 7.9%) 5 weeks after bilateral transplantation into the STN compared to the control value before grafting (p<0.05) (Fig. 2B).

Figure 2: Relative means and SEM of pentylenetetrazole (PTZ) seizure thresholds for induction of (A) myoclonic twitch and (B) clonic seizure before and at different times after bilateral grafting of porcine neuronal progenitor cells into the subthalamic nucleus (STN) of rats immunosuppressed with cyclosporine A (CsA+Graft), neonatally tolerance-induced (Tol+Graft), or without immunomodulation (Graft), as well as in immunosuppressed rats without cell transplantation (CsA). Control seizure thresholds were set to 100%. Sample sizes are given within the bars. Seizure thresholds of the clonic seizure were significantly increased in all groups receiving cell grafts after 10/11 days (10/11 d) compared to control values and compared to non-grafted rats. Note that 5 weeks (wk) after grafting, only tolerance-induced rats showed significantly increased seizure thresholds. Mo, months. (Differences within each group over time, *p < 0.05; **p < 0.01, ***p < 0.001; Intergroup differences, "p < 0.05, "p < 0.01; one-way ANOVA and Dunnett’s posttest).
In contrast to clonic seizure thresholds, the thresholds for induction of myoclonic twitches were not robustly increased by grafting pNPCs into the STN. Increases of the myoclonic twitch seizure thresholds by more than 10% at 10/11 days after grafting were only observed in the Tol+Graft group (116.6 ± 5.5%) and in the Graft group (113.2 ± 4.2%), the latter of which reached statistical significance (p < 0.05) (Fig. 2A).

Even unilateral transplantation of pNPCs into the STN raised the mean clonic seizure thresholds 10/11 days after grafting in rats of the Graft group (141.7 ± 6.7%; p < 0.01) and of the CsA+Graft group (146.6 ± 9.9%; p < 0.01) (not illustrated). Additionally, at that time clonic seizure thresholds of both groups were significantly higher than in the CsA group (p < 0.01 each). Thresholds for induction of first myoclonic twitch were neither altered over time, nor were they different between the groups after unilateral grafting into the STN. Due to low animal numbers, the seizure thresholds of rats in which grafts were located bilaterally outside the STN could not be statistically analyzed (data not shown).

4.4.4 Behavioral Tests

Only rats, in which pNPCs were grafted bilaterally into the STN, were included in the statistical analyses of body weight, body temperature, and behavioral parameters investigated in the OF and EPM, so that the animal numbers for evaluation of these parameters were: Graft group, n = 13; CsA+Graft group, n = 13; Tol+Graft group, n = 14.

4.4.4.1 Body Weight and Body Temperature

During the first behavioral test day (before cell grafting; Fig. 1), rats had a mean body weight of 217.5 ± 1.95 g. Since rats were randomly distributed to the different study groups, body weights did not differ significantly between the groups (p = 0.277). During the second behavioral test day, i.e. about 2 weeks after cell grafting, the body weight of rats significantly increased in all animal groups compared to control values (p < 0.001 in all groups, Fig. 3A). There were no intergroup differences in body weight.

The mean body temperature of the rats was 38.2 ± 0.07°C at the first behavioral test day, without significant differences between the groups (p = 0.606). After grafting, the body temperature decreased in animals of the Graft group (37.8 ± 0.1°C) and the CsA+Graft group (37.3 ± 0.1°C) compared to control value (p < 0.01 and p < 0.001, respectively). No significant
drop in body temperature was observed in the Tol+Graft group (37.9 ± 0.1°C). Intergroup comparisons revealed, that 2 weeks after grafting the body temperature in the CsA+Graft group was significantly lower than in the Graft (p < 0.01) and Tol+Graft group (p < 0.001) (Fig. 3B).

4.4.4.2 Behavior in the Open Field

In order to detect putative adverse effects of the cell transplantation or the different immunomodulatory protocols, we conducted a set of behavioral observations including a hyperexcitability test, and we analyzed the abdominal muscle tone and counted the number of feces boli. None of these parameters revealed significant differences between the study groups or within each group before and 2 weeks after grafting of pNPCs (data not illustrated). Only the occurrence of diarrhea significantly increased after 2 weeks CsA treatment in comparison to control value (p < 0.05) and compared to tolerance-induced rats (p = 0.05) (Fig. 3C).

4.4.4.3 Behavior in the Elevated Plus Maze

In the EPM, we found the total distance moved and the movement velocity to be significantly reduced (p < 0.05 both) 2 weeks after grafting in rats treated with CsA.
(CsA+Graft group) compared to the control value before grafting (Fig. 4A-B). The following parameters were therefore normalized to 10 m distance moved: Number of entries and time spent in the closed arms, open arms, and center. The time spent in the open arms was significantly reduced 2 weeks after grafting in rats of the Graft group (p < 0.05) and the CsA+Graft group (p < 0.01), the latter of which also spent more time in the closed arms (p < 0.05) (Fig. 4C and E).

Figure 4: Means and SEM of parameters investigated in the elevated plus maze test before (control) and 2 weeks after grafting of porcine neuronal progenitor cells bilaterally into the STN. Sample sizes were n = 13 for rats grafted without immunomodulation (Graft), n = 13 for rats grafted and immunosuppressed with cyclosporine A (CsA+Graft), and n= 14 for rats grafted and neonatally tolerance-induced (Tol+Graft). Rats treated with CsA showed a reduction in (A) the total distance moved and (B) the movement velocity 2 weeks after grafting compared to control values. Therefore, the following parameters were normalized to 10 m distance moved: time spent in open and closed arms, and entries into open and closed arms. Two weeks after grafting, CsA-treated rats showed a decrease in (C) time spent in open and (E) closed arms, (G) duration of head dipping, (H) number of head dips, and (J) number of rearing in closed arms. The parameters (D) entries into open and (F) closed arms, (I) duration of rearing in closed arms, and (K) latency to first entry into open arms were not altered over time or between groups. (Differences between the time-points within each group, *p<0.05; **p<0.01, paired t-test; Intergroup differences, °p<0.05, one-way ANOVA and Dunnett’s posttest).
The number of entries into the open and closed arms, respectively, did not differ between the groups or over time (Fig. 4D and F). Animals of the CsA+Graft group not only showed a decreased duration of dipping 2 weeks after grafting compared to the control value ($p < 0.05$), but also compared to animals of the Tol+Graft group ($p < 0.05$) (Fig. 4G). Additionally, the number of dipping was reduced 2 weeks after grafting in the CsA+Graft group ($p < 0.05$) (Fig. 4H). Even though the duration of rearing in the closed arms did not change significantly over time, the number of rearing was lower 2 weeks after grafting in animals of the Graft group ($p < 0.05$) and in animals of the CsA+Graft group ($p < 0.01$) (Fig. 4I and J). The latency of first entrance in one of the open arms of the maze (Fig. 4K), the number of entries into the center, the time spent in the center, and the number and duration of rearing in the center and in the open arms did not change over time and were not different between groups (data not illustrated).

### 4.5 Discussion

The present data show that the neonatal induction of tolerance is a safe and feasible immunomodulatory strategy for grafting pNPCs in experimental epilepsy research: (1) Grafting pNPCs into the STN of tolerance-induced rats resulted in clearly extended anticonvulsant effects compared to CsA-treated or non-immunomodulated rats. Furthermore, (2) the neonatal induction of tolerance did not cause any obvious adverse effects, whereas (3) a pharmacological immunosuppression with CsA caused diarrhea, a reduced body temperature, and a decreased locomotion and exploration behavior of the animals.

#### 4.5.1 Anticonvulsant Efficacy of pNPCs Grafted into the STN

Confirming recent preliminary data of our group (Backofen-Wehrhahn et al., manuscript in preparation), grafting in vitro cultured mesencephalic pNPCs into the STN of rats caused anticonvulsant effects in the present study. These findings are in line with a recent transplantation study of our group, in which we showed for the first time that grafting GABAergic cell lines into the STN induced anticonvulsant effects in rats (Handreck et al., 2014).
In the present study, anticonvulsant effects were observed in all treatment groups after grafting into the STN, including animals without immunomodulation. This was rather unexpected, because a xenotransplantation of porcine tissue has often been shown to cause a strong T-cell mediated rejection response and also a humoral immune response and an activation of the complement system (Pakzaban and Isacson, 1994; Brevig et al., 2000; Cascalho and Platt, 2001; Barker and Widner, 2004). In a rat model of Parkinson’s disease, Barker et al (2000) observed that pNPCs from ventral mesencephalon grafted into the striatum of non-immunosuppressed rats maturated to dopaminergic cells and showed porcine-specific neurofilament fibers until 10 days after transplantation despite the lack of immunomodulation. Markers for an ongoing immune response were clearly detectable. Only 21 days after grafting, the cells were rejected (Barker et al., 2000). This might explain the initial anticonvulsant effect observed in the present study 10/11 days after grafting pNPCs in non-immunomodulated rats. Similar to the lack of cell survival 3 weeks after grafting in the study of Barker and colleagues (2000), we could not observe anticonvulsant effects 3 weeks after grafting in non-immunomodulated rats, indicating that the pNPCs have been rejected at this time.

CsA-treated animals showed similar initial anticonvulsant effects as non-immunomodulated animals. Following discontinuation of the CsA treatment after 15 days, the seizure thresholds went back to the pre-transplantation value 3 weeks after grafting, again indicating a rejection of the transplanted pNPCs in the absence of the pharmacological immunosuppression. Three months after grafting we could not observe obvious signs of host tissue reactions at the transplantation site in the different groups. At that time, the graft rejection process probably was completely terminated. However, specific immunohistochemical staining to detect cellular immune responses will be conducted in further investigations.

In contrast to CsA-treated rats or rats without immunomodulation, tolerance-induced animals showed a delayed increase of seizure thresholds 5 weeks after grafting in addition to the initial elevation of chronic seizure thresholds 10/11 days after transplantation. It has to be further investigated using immunohistological methods, if the delayed anticonvulsant effect is due to a successful integration of grafted pNPCs protected by tolerance induction. Although the neonatal induction of tolerance clearly prolongs the anticonvulsant effects of
grafting pNPCs into the STN, the observed effects were still transient. The strategy of neonatal tolerance induction has been reported to promote graft survival of human and mice neuronal precursor cells up to 12 weeks after transplantation into the striatum of rats (Kelly et al., 2009). Therefore, upcoming immunohistochemical investigations have to show whether the grafted pNPCs were rejected by the host or if the cells survived until the end of the study but no longer act anticonvulsant due to different reasons such as a reduced GABA-expression.

Porcine ventral mesencephalic precursor cells are typically used for cell replacement approaches in experimental Parkinson’s disease models and in clinical trials involving human Parkinson’s disease patients (HogenEsch et al., 2000; Larsson et al., 2000; Larsson and Widner, 2000; Larsson et al., 2001; Wennberg et al., 2001; Armstrong et al., 2003). These cells, when grafted as primary cells, develop a dopaminergic phenotype after transplantation (Armstrong et al., 2003). However, as shown by Wegner and colleagues, cells isolated from the whole mesencephalon develop into GABAergic cells during in vitro culturing (Wegner et al., 2008; Wegner et al., 2009), rendering them an interesting cell type for neurotransplantation in epilepsy.

The observed anticonvulsant effects after grafting pNPCs in the present study are thus most likely based on a GABAergic differentiation of these cells resulting in an inhibition of the STN. As indicated by several pharmacological studies, an inhibition of the STN reduces the excitatory input to the SNr and thus leads to an indirect inhibition of the SNr, thereby causing anticonvulsant effects (Deransart et al., 1996; Velísková et al., 1996; Deransart et al., 1998; Dybdal and Gale, 2000; Gale et al., 2008; Bröer et al., 2012). Furthermore, the STN has additional connections to other brain regions such as the cortex, the entopeduncular nucleus, and the pedunculopontine nucleus (Dybdal and Gale, 2000; Degos et al., 2008; Gale et al., 2008; Bröer et al., 2012). This might explain in part the anticonvulsant efficacy of unilaterally grafted pNPCs into the STN of rats. Similarly, unilateral transplantations of GABAergic cell lines (Handreck et al., 2014) and unilateral microinjections of muscimol (Velísková et al., 1996) into the STN were shown to induce anticonvulsant effects as well. Velísková and colleagues hypothesized that unilaterally misplaced muscimol diffuses from surrounding regions into the STN, thereby adding to the effects from the correctly placed side. For neurotransplantations this is rather unlikely, because the bilateral transplantation
of GABAergic cell lines into regions outside the STN was not anticonvulsant in our previous study (Handreck et al., 2014). On the other hand, neural precursor cells in general have a high potential of migration and were shown to migrate up to several millimeters into other brain regions after transplantation (Olsson et al., 1997; Anderson et al., 2001; Alvarez-Dolado et al., 2006). Further studies should clarify this issue.

4.5.2 Adverse Effects caused by CsA treatment

In CsA-treated rats, a wide range of unwanted adverse effects was detected. In contrast, we did not observe any adverse effects in animals that were neonatally tolerance-induced. Although all treatment groups showed a normal weight gain after transplantation, diarrhea was observable in animals treated with CsA, what is in line with previous findings (Handreck et al., submitted). Since CsA is known to damage the gastro-intestinal tract and to increase the risk of infections (Rosendal et al., 2005), this adverse effect was expected. Furthermore, all treatment groups had a more or less reduced body temperature two weeks after grafting, which may be explained by the fact that the animals had a reduced stress level when being in the OF for the second time. However, the body temperature of CsA-treated animals was even lower than in the other groups. These findings are also in line with our recent study evaluating different CsA treatment regimens with respect to their safety and feasibility (Handreck et al., submitted). As reported previously, CsA interferes with the thermoregulation and can lead to hypothermia (Dantzer et al., 1987).

Apart from these physiological interferences, CsA is known to be neurotoxic in human patients and in rodents (Famiglio et al., 1989; Gijtenbeek et al., 1999; Bechstein, 2000; Wijdicks, 2001; Serkova et al., 2004). CsA can cross the blood-brain-barrier by causing direct endothelial damage (Lane et al., 1988; Bronster et al., 1999; Dohgu et al., 2000; Wijdicks, 2001) or by inhibiting the function and expression of P-glycoprotein (Kochi et al., 1999; Syvänen et al., 2006). The reduced locomotion and horizontal as well as vertical exploration of CsA-treated rats observed in the present study might be related to the neurotoxic effects of the CsA treatment. Alterations in the activity of calcineurin or regulators of calcineurin (Liu et al., 2013), oxidative stress, and inhibition of mitochondrial function might induce neurotoxic effects (Christians et al., 2004; Klawitter et al., 2010). It is interesting to note that other studies reported spontaneous hyperactivity in normal rats after CsA injection.
(Borlongan et al., 1995; Borlongan et al., 1999). However, this discrepancy may be explained by the use of different CsA preparations and gender in their investigations and our study. Additionally, the reduced locomotion and exploration may indicate development of increased anxiety-related behavior or depression. As reported by Chen and colleagues (2010), depressive symptoms can be induced by a down-regulation of the brain-derived neurotropic factor and its receptor tyrosine kinase receptor B in the hippocampus of rats after chronic treatment with CsA. However, the reduced locomotion and exploration could also be based on the gastrointestinal problems of the animals, thus resulting in a general discomfort.

4.6 Conclusion

The present findings indicate that the neonatal induction of tolerance can be preferred over a pharmacological immunosuppression with CsA. In line with previous studies (Kelly et al., 2009; Zhang et al., 2013), this immunomodulatory strategy seems to promote neural graft survival and clearly prolonged the anticonvulsant effects induced by transplantation of pNPCs into the STN, without causing adverse effects. However, the observed effects were transient and other studies even reported a failure of this strategy (Janowski et al., 2012; Roberton et al., 2013). The efficacy of the neonatal tolerance induction to prevent graft rejection seems to be dependent on several factors, such as the host species, donor cell type, and maybe the target region within the host. Our promising data on neonatal induction of tolerance and the use of pNPCs for xenotransplantation studies in epilepsy research merit further investigations.

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4.7 References


STUDY 3: PORCINE CELL GRAFTING AND INDUCTION OF TOLERANCE


5 GENERAL DISCUSSION

The main findings of the studies presented in this work are (1) neurotransplantation of GABAergic cell lines and pNPCs into the STN of rats induced anticonvulsant effects, which (2) were observed even after unilateral transplantation into the STN, and (3) were in part stronger and longer-lasting than previously observed after grafting the same GABAergic cell lines into the SNr using a different epilepsy model (Nolte et al., 2008). Furthermore, we showed that (4) a neonatal induction of immunological tolerance towards pNPCs prolonged anticonvulsant effects induced by grafting of pNPCs into the STN. (5) The pharmacological immunosuppression with different preparations of CsA did not robustly affect seizure thresholds in the acute PTZ seizure threshold test and in the chronic amygdala-kindling model, but (6) was associated with reversible adverse effects such as reduced locomotion and diarrhea. These are the first studies showing anticonvulsant effects of neural transplantations into the STN in epilepsy research.

5.1 Anticonvulsant Efficacy of GABAergic Cell Lines and Porcine Neuronal Precursor Cells Transplanted into Rats

5.1.1 Anticonvulsant Effects of a Bilateral Neurotransplantation into the Subthalamic Nucleus

For neural transplantation into the STN, we used GABAergic cell lines from rats and pNPCs. Both cell types caused clear anticonvulsant effects after grafting into the STN of rats. It can be assumed that these anticonvulsant effects are at least in part the result of an indirect inhibition of the SNr. In vitro studies indicated that the immortalized cells produce and release GABA (Giordano et al., 1993; Conejero-Goldberg et al., 2000) and also the pNPCs were shown to develop a GABAergic phenotype when cultivated prior to transplantation (Wegner et al., 2008; Wegner et al., 2009). After transplantation, the cells can be thought to inhibit STN neurons, resulting most likely in an inhibition of downstream SNr neurons due to reduced excitatory input from the STN (Dybdal and Gale, 2000; Löscher et al., 2008). A recent study of our group, in which vigabatrin was microinjection bilaterally into the brain in the PTZ seizure threshold test, showed that a pharmacological inhibition of the STN
induced stronger anticonvulsant effects than the microinjection into the SNr or a systemic administration of vigabatrin (Bröer et al., 2012). We now could extend these recent findings. The bilateral transplantation of the GABAergic cell line M213-2O into the STN induced stronger anticonvulsant effects than a transplantation into the SNr, although a direct comparison is hampered by the fact that in the previous SNr study a different rat model was used (Nolte et al., 2008). It is conceivable that an inhibition of the STN enhances the anticonvulsant effects via mechanisms that are not only mediated via the SNr, but additionally via glutamatergic projections to the cortex, the entopeduncular nucleus, and the pedunculopontine nucleus (Fig. 5) (Dybdal and Gale, 2000; Degos et al., 2008; Gale et al., 2008; Bröer et al., 2012; Gernert, 2013; Handreck et al., 2014).

Fig. 5: Schematic illustration of putative mechanisms underlying the anticonvulsant effects of a GABAergic cell transplantation (GABAergic cell lines and porcine neuronal precursor cells) into the subthalamic nucleus (STN). The indirect inhibition of the substantia nigra pars reticulata (SNr) by inhibiting the STN with GABA-producing cell grafts leads to a disinhibition of downstream structures and anticonvulsant effects. Additionally, the effect of inhibiting the STN is suggested to be additionally mediated via connections to other brain regions. GABA, γ-aminobutyric acid.

5.1.2 Anticonvulsant Effects of an Unilateral Transplantation into the Subthalamic Nucleus

Interestingly and unexpectedly, even unilateral transplantation of GABAergic cell lines and pNPCs, respectively, caused anticonvulsant effects at least 10 to 11 days after grafting. The anticonvulsant effects of unilaterally grafted M213-2O cells were even longer-lasting (3
weeks) than in the previous study of Nolte at al. (2008), in which the cells induced an elevation of the generalized seizure threshold for only one week after grafting into the SNr in kindled rats. This could be attributed to the STN as target region or to the PTZ seizure threshold test for evaluating the effects, because this acute seizure model is especially sensitive for manipulations of the GABAergic system (Löscher, 2009). As described above, the more pronounced anticonvulsant effects after grafting into the STN are likely not only mediated by inhibiting the SNr, but also by connections to other brain regions such as cortex, entopeduncular nucleus, and pedunculopontine nucleus (Fig. 5) (Dybdal and Gale, 2000; Degos et al., 2008; Gale et al., 2008; Bröer et al., 2012; Gernert, 2013; Handreck et al., 2014).

Comparable to the here presented results, an unilateral microinjection of muscimol into the STN was sufficient to induce anticonvulsant effects in the flurothyl model (Velísková et al., 1996). However, in that study muscimol was microinjected bilaterally, but with correct placement within the STN in only one hemisphere. The group suggested that in the hemisphere with the misplacement, muscimol may diffuse to the STN from regions surrounding the STN, thereby causing anticonvulsant effects together with the hemisphere with correct drug placement. For neurotransplantations this is rather unlikely, even more so as we showed that a bilateral transplantation of the GABA-producing cell lines in regions outside, but close to the STN (cerebral peduncle, zona incerta) did not induce anticonvulsant effects (Handreck et al., 2014). However, neural precursor cells indeed have a high potential of migration even after grafting into the adult brain (Olsson et al., 1997; Anderson et al., 2001; Alvarez-Dolado et al., 2006).

It cannot be excluded that part of the observed anticonvulsant effects is a result of host tissue reactions (as observed after grafting Clone 4 cells) or graft rejections (as assumed after grafting pNPCs in non-immunomodulated rats). Indeed, the occurrence of absence seizures was reduced after bilateral lesions of the STN with kainic acid in a genetic rat model for absence epilepsy (Vercueil et al., 1998). However, Blandini et al. (2006) and Shehab et al. (2006) did not report anticonvulsant effects in response to STN lesions.

In general, we could confirm the initial hypotheses that the bilateral (and also unilateral) transplantation of GABA-producing cell lines as well as pNPCs into the STN results in anticonvulsant effects in an acute seizure model and that these effects are even stronger
and more long-lasting than after grafting the same GABAergic cell lines into the SNr, using a different epilepsy model. Due to the fact that a transplantation of non-GABAergic cells into the STN and a transplantation outside the STN did not induce anticonvulsant effects, we could also prove that the observed effects are not only specific for the GABAergic cell lines and pNPCs, but also for the STN as transplantation site.

5.1.3 Lack of Long-lasting Anticonvulsant Effects

Similar to previous studies targeting the SNr, the observed anticonvulsant effects after unilateral and bilateral grafting of GABAergic cell lines and pNPCs into the STN were only transient, even though the GABAergic cells could still be identified up to at least 5 weeks after grafting via staining with bisbenzimide. This lack of long-lasting effects does most likely not result from tumor formations. The GABAergic cell lines are of striatal origin from rats and were immortalized using a temperature-sensitive oncogene (tsA58 of the SV40 large T antigen). As a result, the cells proliferate at a temperature of 33°C and stop dividing at 39°C, due to a conformational change of the large T protein (Giordano et al., 1993; Truckenmiller et al., 1997). Therefore, the cells have a low risk of tumor formation. Even though the cells were cultured at 33°C and only reached a warmer environment when grafted into the host tissue (37°C), we could not observe any obvious signs of tumor formation. This is in line with previous findings, in which staining for the tumor marker Ki67 did not indicate tumor formations after grafting of these cell lines into the rat brain (Nolte et al., 2008). Also for the transplanted pNPCs, tumor formations were not obvious. These cells were cultivated as spheres without serum, which eliminates non-neuronal cells and thus lowers the risk of a teratoma formation, as was shown with human precursor cells (Reubinoff et al., 2001).

Different conceivable reasons can be discussed for the transience of the anticonvulsant effects. A tolerance towards the additionally produced and released GABA may have developed, meaning that either the GABA synthesis could be reduced over time or the expression of postsynaptic GABA receptors in host tissue could be down-regulated. Furthermore, an insufficient supply with nutrients and oxygen, the limited space within the densely packed STN, and an insufficient removal of metabolites may cause a continuous cell death of the grafts. It was described earlier that the number of neocortical GABAergic cells grafted into the striatum decreased over time (Bragin et al., 1993). In future studies, we will
determine the graft volume after transplantation (Rosen and Harry, 1990) in order to estimate the survival rate of the grafted cells.

5.2 Necessity, Efficacy, and Safety of an Immunomodulation for Neurotransplantations in Experimental Epilepsy Research

The brain has long been regarded as an immunologically privileged site, because of the isolation of the brain by the blood-brain-barrier and the presence of active efflux transporters such as P-glycoprotein. Furthermore, a lack of antigen-presenting cells and lymphatic vessels in the brain has been postulated. However, it is now known that activated lymphocytes are able to cross the blood-brain-barrier. Additionally, in neurotransplantation studies the transplantation procedure inevitable causes damage of the blood-brain-barrier (Barker and Widner, 2004). Especially for xenotransplantations, an immunomodulation is therefore essential to prevent graft rejection.

5.2.1 Tissue Reactions after Neurotransplantation of hGAD-overexpressing Cells

The transplantation of the hGAD-overexpressing cell line Clone 4 caused tissue reactions and gliosis with different degrees of manifestations, which were not induced by M213-2O and non-GABAergic 121-1I cells. This was observed in the study presented here (Handreck et al., 2014) and in the study by Nolte et al. (2008). In previous studies using other animal models, this reaction was not observed (Ross et al., 2002; Castillo et al., 2006; Castillo et al., 2008). Therefore, Nolte et al. (2008) suggested that the graft rejections can be in part related to the kindling model used in their study, because the kindling process itself has been shown to induce microgliosis in the SNr (Ebert et al., 1996).

However, other factors must be additionally involved, because in our study tissue reactions to Clone 4 cells could also be observed in naïve animals. A host immunoreaction against the human GAD67, which is expressed by this cell line, may be a likely explanation. As shown in diabetes research, this protein is a strong immunogen and can induce autoimmune diseases in patients (Ludvigsson, 2009). Increased levels of antibodies against GAD were also detected in some epilepsy patients (Vianello et al., 2002; Errichiello et al., 2009; Liimatainen et al., 2010; Bien and Scheffer, 2011). Even though the Clone 4 cell line was originally isolated from
the rat, meaning that an allotransplantation was performed in our study, the cells were transfected and express the human antigen. Due to the overexpression, a leakage of hGAD is conceivable. Thus, this antigen can be detected as non-self by the host’s immune system. Likewise, the Epstein-Barr virus nuclear antigen 1 and the hygromycin resistance gene expressed by Clone 4 cells could be further triggers for the immunoreaction, because they represent further differences to the parent cell line M213-2O, which did not induce tissue reactions after grafting.

5.2.2 Cyclosporine A does not Robustly Influence Seizure Thresholds

In humans, CsA is known to cause neurotoxicity including an increased susceptibility to seizures (Bechstein, 2000; Serkova et al., 2004). On the other hand, CsA has neuroprotective properties and thus has been suggested as a promising treatment approach for stroke patients (Osman et al., 2011). In order to conduct xenotransplantation studies in experimental epilepsy models under immunosuppressive treatment, the effect of CsA on the respective models has to be properly evaluated. In fact, proconvulsant but also anticonvulsant effects of CsA were observed in several different animal models for epilepsy (Racusen et al., 1988; Famiglio et al., 1989; Racusen et al., 1990; Moia et al., 1994; Asanuma et al., 1995a; Asanuma et al., 1995b; Shuto et al., 1999; Reubinoff et al., 2001; Fujisaki et al., 2002; Homayoun et al., 2002; Santos and Schauwecker, 2003; Setkowicz et al., 2004; Yamauchi et al., 2005; Setkowicz and Ciarach, 2007; Jung et al., 2012). However, since these studies used different application routes, doses, and preparations of CsA, as well as treatment regimens and epilepsy models, there is no definite immunosuppression protocol, which can be safely used in epilepsy models. We therefore comprehensively compared the effects of different CsA treatment regimens on seizure thresholds in the PTZ seizure test and the kindling model, without observing robust pro- or anticonvulsant effects.

This was unexpected, because several different mechanisms for pro- or anticonvulsant effects of CsA are discussed in the literature. For example, it could be demonstrated that nitric oxide has proconvulsant properties (Proctor et al., 1997; Han et al., 2000) and is increased in animal models for epilepsy (Elmer et al., 1996). During treatment with CsA, the nitric oxide production is raised dose-dependently due to the inhibition of calcineurin in the hippocampus and thereby inhibits GABAergic neurotransmission, resulting in proconvulsant
effects (Fujisaki et al., 2002). This mechanism is supposed to be closely related to adverse
effects like tremor (Shuto et al., 1999). On the other hand, it is argued that the inhibition of
calcineurin by CsA reduces the neuronal excitability by enhancing the expression of GABA_A-
receptors (Martina et al., 1996; Jung et al., 2012). Furthermore, CsA may act anticonvulsant
by decreasing the activity of nitric oxide synthetase (Homayoun et al., 2002) or by increasing
the excitability of hippocampal neurons (Wang and Wang, 2002).

Nevertheless, in animals treated subcutaneously with Sandimmune® (Novartis
Pharmaceuticals), no effects on seizure thresholds could be observed. Accordingly,
compared to the other treatment groups, the CsA whole blood concentration was the
lowest. In contrast, the intraperitoneal treatment with 10 mg/kg Sandimmune® induced the
highest CsA whole blood levels, thus showing the most effective resorption. Together with
the unaffected PTZ and kindling seizure thresholds during and after the immunosuppressive
treatment, the i.p. application of 10 mg/kg Sandimmune® seems to be the most feasible and
secure method for a chronic immunosuppression, when using the PTZ seizure test or the
amygdala-kindling model in rats.

5.2.3 **Adverse Effects of Treatment with Cyclosporine A**

Although we could establish an appropriate CsA treatment protocol, which is sufficiently
safe for the use in the PTZ seizure threshold test and the kindling model, it nevertheless
cause different adverse effects for the animals, which mainly comprised (1) diarrhea, (2)
tremor, (3) a decreased body temperature, (4) reduced locomotion, and (5) diminished
horizontal and vertical exploration. As hypothesized initially, rats immunomodulated by
neonatal induction of tolerance did not show any of the adverse effects observed in CsA-
treated rats.

Diarrhea was an expected adverse effect in the CsA-treated animals. Firstly, especially the
pure CsA solutions contained a high concentration of polyoxyethylene castor oil, and
secondly, it is known that CsA damages the gastro-intestinal tract and increases the risk of
infections (Rosendal et al., 2005). The mechanisms of CsA neurotoxicity probably underlying
most of the other adverse effects are not fully understood and multifactorial causes can be
discussed. Despite low penetration via the blood-brain barrier, high concentrations of CsA
and also CsA metabolites were found in the cerebral fluid in CsA-treated humans (Bronster
et al., 1999). This is most likely the result of an increased permeability of the blood-brain barrier due to direct endothelial damage (Lane et al., 1988; Dohgu et al., 2000; Wijdicks, 2001) or inhibition of the function and expression of P-glycoprotein (Kochi et al., 1999; Syvänen et al., 2006). Neurotoxic effects during CsA treatment may partially be caused by alterations in the activity of calcineurin and regulators of calcineurin (Liu et al., 2013). In vitro experiments suggest that changes in the metabolism of glucose, caused by oxidative stress and inhibition of mitochondrial function, may also induce neurotoxic effects (Christians et al., 2004; Klawitter et al., 2010). In human patients, neurotoxic effects could be linked to hypertension, liver dysfunction, and changes in the white matter (Bechstein, 2000; Thompson et al., 2009). The development of tremor is closely related to proconvulsant effects and most likely based on changes in the GABAergic neurotransmission (Shuto et al., 1999). Tremor was shown to be more frequent at high doses of CsA but is nevertheless often mild in humans (Gijtenbeek et al., 1999). Similarly, we also observed only mild tremor in the CsA-treated animals. Noteworthy, vehicle-treated animals also showed adverse effects. In vitro studies showed that polyoxyethylene castor oil itself can induce neurotoxic effects including axonal swelling, degeneration and demyelination even at low doses (Windebank et al., 1994).

With regard to the drop in body temperature we observed in CsA-treated rats, CsA is known to interfere with thermoregulation and leads to hypothermia (Dantzer et al., 1987). It should be noted that the observed reduction of body temperature as well as the tremor and the reduced locomotion and exploration could also be related to a general indisposition caused by gastrointestinal problems of the animals. Additionally, depressive symptoms were reported after chronic treatment with CsA, maybe induced by a down-regulation of the brain-derived neurotropic factor and its receptor tyrosine kinase receptor B in the hippocampus (Chen et al., 2010).

Tolerance-induced rats did not show any physical impairments or behavioral alterations. Therefore, at least regarding the adverse effects, the induction of neonatal tolerance is preferable to a chronic CsA treatment as immunomodulatory strategy in neurotransplantation studies.
5.2.4 **Comparison of Immunomodulatory Strategies to Promote and Prolong the Anticonvulsant Efficacy of Grafted Porcine Neuronal Precursor Cells**

In line with recent preliminary findings from our group (Backofen-Wehrhahn et al., 2014), the transplantation of pNPCs into the STN caused anticonvulsant effects ten to eleven days after grafting in all groups receiving these cells, i.e. in CsA-immunosuppressed rats, in neonatally tolerance-induced rats, and in non-immunomodulated rats. Due to major antigens, like galactose-α-1,3-galactose, xenotransplantations of porcine tissue typically cause strong humoral rejection responses, which include an activation of the complement system and natural killer cells (Pakzaban and Isacson, 1994; Brevig et al., 2000; Cascalho and Platt, 2001; Barker and Widner, 2004), even though cultivated pNPCs showed a lower immunogenic potential than primary tissue (Harrower et al., 2006).

Accordingly, in contrast to transiently immunosuppressed rats or rats without immunomodulation, tolerance-induced animals showed an elevation of seizure thresholds up to 5 weeks after grafting. However, similar to recent findings from other groups (Janowski et al., 2012; Roberton et al., 2013), the induction of immunological tolerance towards pNPCs was not sufficient to promote long-term anticonvulsant effects up to three months after grafting the pNPCs. In further immunohistochemical investigations, we have to analyze whether the grafted porcine tissue was immunorejected or if the cells survived until the end of the study period 3 months after grafting, as indicated by Kelly et al. (2009). Therefore, we will measure the graft volumes in all groups transplanted with pNPCs.

Using a rat model of Parkinson’s disease, Barker et al. (2000) showed that porcine ventral mesencephalic cells grafted into the striatum of non-immunosuppressed rats were rejected during the first days or weeks after transplantation. Until 10 days after transplantation, the cells maturated to dopaminergic cells and even showed porcine-specific neurofilament fibers even though an immune response could already be observed, which led to a complete loss of porcine cells 21 days after grafting (Barker et al., 2000). This may explain the initial elevation of seizure thresholds we observed 10/11 days after transplanting pNPCs in rats that did not receive an immunomodulatory treatment. At this time-point, a sufficient number of grafted cells may have been alive to induce an anticonvulsant effect. Similar to the lack of surviving cells 3 weeks after grafting into the striatum as described in the study of
Barker and colleagues (2000), we could not observe anticonvulsant effects 3 weeks after grafting pNPCs, indicating that the grafted cells have been rejected at this time. As expected, in animals immunosuppressed with CsA over a period of 15 days, anticonvulsant effects were also not observable anymore 3 weeks after grafting. At that time, the seizure thresholds were back to the control level, again indicating a rejection of the transplanted pNPCs in the absence of the pharmacological immunosuppression. Noteworthy, the initial anticonvulsant effects in animals treated with CsA were not stronger than in non-immunomodulated rats, in which the immune response can be expected to be already activated and to induce some degree of graft rejection. The xenografts of immunosuppressed animals are probably also slightly rejected despite treatment with CsA. Indeed, a conventional pharmacological immunosuppression with CsA as monotherapy does not entirely prevent graft rejection and immune responses (Larsson et al., 2000; Wennberg et al., 2001). Despite immunosuppression, an infiltration with T-cells and macrophages was reported for rats that received porcine precursor cell grafts into the striatum (Larsson et al., 2000). Clinically, a triple therapy with CsA, azathioprine, and prednisolone in Parkinson’s disease patients has been shown to sufficiently prevent immune reactions without causing adverse effects (Widner, 1998; Barker and Widner, 2004). In animal models, a combined therapy with CsA and prednisolone also reduced the immune response (Wennberg et al., 2001).

5.3 Localization of the Neural Grafts – Methodical Issues

In general, the localization of the grafts was verified in 40 µm brain sections using thionine staining. Some of the GABAergic grafts were preincubated with bisbenzimidide, which facilitated the exact localization. The staining with bisbenzimidide was reported not to affect the cell growth or survival rate (Castillo et al., 2008). Furthermore, it is unlikely that astrocytes surrounding the grafted cells were also labeled with bisbenzimidide by transcellular transport or apoptotic DNA release (Aleksandrova et al., 2002; Lee et al., 2003; Castillo et al., 2006).

The pNPCs were transfected to express the fluorescent protein tdTomato (Garrels et al., 2011; Garrels et al., 2012). However, it was very difficult to detect grafted cells on the basis of their fluorescence. This could be due to the fact that the intensity of the fluorescent signal
decreased during the process of tissue preparation or based on a low survival rate of the cells. Therefore, in further histological analyses we plan to perform specific immunostainings, e.g. against the porcine-specific neuronal filament 70 (NF70), in order to measure the graft volume and evaluate the survival of the xenografts (Rosen and Harry, 1990). Furthermore, we want to determine the portion of differentiation of pNPCs to GABAergic cells, dopaminergic cells, or glial cells. Since the brain sections prepared for immunohistochemistry are relatively thin, information about the migration, integration, and interaction of the grafted pNPCs within the host tissue is difficult to obtain. As previously reported, neural progenitors can migrate up to several millimeters within the host brain and then functionally integrate into the existing network (Olsson et al., 1997; Anderson et al., 2001; Alvarez-Dolado et al., 2006; Anderson and Baraban, 2012; Hovakimyan et al., 2012). It would be elegant to image the transplantation site without slicing the brain and thereby destroying the complex network between host tissue and grafted cells.

With CLARITY, Chung and colleagues (2013) developed a technique to obtain high-resolution, three-dimensional images of intact, unsliced tissue. With this method, they were able to transform a whole mouse brain into a completely transparent hydrogel-hybridized form, which enabled them to image the whole brain and gain information about the three-dimensional structure and (applying immunohistochemistry) specific cellular networks. According to the authors, this technique can also be used for various tissues including small tissue and brain slices (Chung and Deisseroth, 2013; Chung et al., 2013). Because a high processing power is required to analyze larger tissues and because this technique is still limited to tissues of the size of a mouse brain, it would be more feasible for the normal routine work to investigate (up to several millimeter) thick brain sections instead of the whole brain.

During a research stay in the lab of Dr. William J. Freed at the National Institutes of Health (National Institute on Drug Abuse, Cellular Neurobiology Research Branch, Section on Development and Plasticity, Baltimore, USA), I adapted the CLARITY protocol for use in small tissue (Tab. 1, Supplements). Therefore, I used 3D cortical organoids that were similar to the ones described by Lancaster et al. (2013). These organoids represent an in vitro model of cortical development and resemble spheres built of human embryonic stem cells at different levels of cortical differentiation (Lee et al., unpublished data, manuscript in preparation).
could successfully adapt the CLARITY protocol and image the complete 3D cortical organoids without prior slicing (Fig. 6, Supplements). Since the cortical organoids have a size of at least one millimeter, this adapted protocol could also be used for clearing thick brain sections, e.g. after grafting pNPCs. For neurotransplantation studies, this technique would be a tremendous enrichment in terms of imaging the grafted cells within the complex host tissue and analyzing their migration and integration in the existing network.

5.4 Conclusions and Outlook

In a proof-of-principle study using GABAergic cell lines, we showed for the first time that the STN is a well suited target region for neurotransplantations in epilepsy research. Likewise, results from xenografting pNPCs into the STN emphasized the promising properties of the STN as a target region in this respect. The fact that the STN is already clinically established as a target region for neurosurgery adds to the advantages of the STN. However, despite long-term survival of grafted cells, the anticonvulsant effects were only transient, comparable to previous findings from graftings into the SNr.

In a first approach to solve this critical issue, we successfully prolonged anticonvulsant effects of pNPCs grafted into the STN by neonatal induction of tolerance for the grafted cells. Further studies are needed to evaluate, if this efficacy could be further prolonged by applying tolerance induction in combination with a pharmacological immunosuppression as a strategy to prevent graft rejection. The use of porcine cells for neurotransplantation clearly merits further studies, because it raises less ethical concerns than the collection of tissue from human fetuses. Even though zoonotic retroviral infections are a possible risk factor of xenotransplantations in humans, in 160 patients suffering from different diseases, no evidence of a retroviral infection was detectable in any of these patients after receiving different kinds of porcine tissue grafts (Paradis et al., 1999). To prevent graft rejection, humanized transgenic porcine tissue expressing the human complement inhibitor CD59 was suggested to be a promising option for successful translation of xenotransplantations into clinic (Cicchetti et al., 2003). Continuing the cooperation with Prof. Dr. H. Niemann (FLI, Mariensee), we will additionally investigate different cell types from humanized pigs for neurotransplantation in experimental epilepsies.
REFERENCES


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102


7 SUPPLEMENTS

Fig. 6: CLARITY method adapted for the use of small tissue.

(A) Time line showing main procedures for clearing the tissue and subsequent immunostaining and preparation for imaging. D, day/days; h, hours; PBST, phosphate buffered saline with triton X-100; RT, room temperature; w, week/weeks.

(B) Shown is a 3D cortical organoid (developed from human embryonic stem cells) in clearing solution in a 1.5 ml tube. At day 1 of passive clearing (upper left) the organoid is easily visible. During the passive clearing, the organoid then becomes more and more transparent. The organoid in the illustrated example is completely transparent at day 12 of clearing (upper right) and can only be visualized when hold directly into light (lower image). Arrows indicate the location of the organoid.

(C) Confocal laser-microscope images of an entire (unsliced) 3D cortical organoid. Shown is a series of images obtained by focusing through the z-axis of the completely cleared organoid (first image, upper row first picture; last image, lower row right picture). The distance between each image of the series is 9.81 µm. The organoid was immunostained against Tuj1 (Neuronal class III Beta-Tubulin; red), a marker for fully differentiated cortical neurons (typically located in the periphery of the 3D organoids), and FoxG1 (green), which is a forebrain progenitor marker (typically located in the center of the organoid). For imaging, the organoid was embedded in low-melting point agarose gel. Scale bar: 200 µm.
### Main procedures

<table>
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<tr>
<th><strong>Original Protocol</strong> (Chung et al., 2013) for whole mouse brains</th>
<th><strong>Modified protocol for 3D cortical organoids</strong></th>
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</table>
| Crosslinker contained in hydrogel solution: | Acrylamide (40%)  
Bisacrylamide (2%)  
| Acrylamide (40%)  |
| Diffusion of hydrogel | Transcranial perfusion with ice cold PBS and ice cold hydrogel solution  
| Dipping into ice cold PBS followed by transfer to ice cold hydrogel solution  |
| Incubation in hydrogel solution | 2-3 days; 4°C  
| 2 days; 4°C  |
| De-gassing | Replacement of oxygen with nitrogen in a desiccation chamber  |
| Hydrogel tissue embedding | Incubation at 37°C; 3 h  |
| Washing with clearing solution  | 24 h at RT  
| 2x for 48 h at 37°C  |
| Electrophoretic tissue clearing (ETC) | Circulation of clearing solution through a ETC chamber with 10-60 V at 37-50°C until tissue is completely clear  
| Incubation in clearing solution at 37°C until tissue is clear (1-3 weeks); periodical change of clearing solution  |
| Passive clearing | /  |
| Washing | 2x for 24 h at RT with PBST  |
| Incubation in primary antibody solution | 2 weeks at 37°C;  
Primary antibody in 0,1% triton X-100 and 0.5 M sodium borate buffer  
| 2 days at RT;  
Primary antibodies (Tuj1, 1:1000; FoxG1, 1:50) in 0,2% triton X-100, 5% serum, 5% BSA and PBS  |
| Washing | 1 week at 37°C;  
with 0,1% triton X-100 in 0.5 M sodium borate buffer  
| 2x for 24 h at RT;  
with PBST  |
| Incubation in secondary antibody solution | 2 weeks at 37°C;  
secondary antibody in 0,1% triton X-100 and 0.5 M sodium borate buffer  
| 2 days at RT;  
secondary antibodies (1:200 each) in 0,2% triton X-100, 2% BSA, and PBS  |
| Washing | 1 week at 37°C;  
with 0,1% triton X-100 in 0.5 M sodium borate buffer  
| 2x for 24 h at RT;  
with PBST  |
| DAPI staining | /  
| 1:5000 in PBST for 2 h followed by washing in PBST for 24 h  |
| Matching refractive indices | Incubation in 80% glycerol or FocusClear; 2 days  
| Incubation in 80% glycerol; 24 h  |
| Tissue embedding | Embedding in a glass chamber filled with 80% glycerol or FocusClear  
| Embedding in low melting point agarose gel (1%; 40-60°C) in a petri dish; ready for imaging when fully polymerized at RT  |

Tab. 1: Differences and analogies of main procedures of CLARITY and immunostaining. h, hours; PBS, phosphate buffered saline; PBST 0,1% triton X-100 in PBS; RT, room temperature; V, volt.
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