

20th Annual Meeting of the Network of European CNS Transplantation and Restoration (NECTAR)

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Dear Colleagues,

this year's Annual Meeting of the Network of European CNS Transplantation and Restoration (NECTAR) will take place from November 25-27, 2010 in Freiburg, Germany. This year, we will celebrate some anniversaries in that NECTAR was founded 20 years ago, and this year's meeting will be the 20th.

Functional restoration in the central nervous system has during recent years attracted increased attention not only from scientists and clinicians worldwide but increasingly also from patient foundations, media and health care systems. And there are clinical trials ongoing with regard to cell replacement, neuroprotection, and gene therapy in different neurological disorders. The local and international organizing committees have composed a programme with leading scientists and clinicians addressing key issues on experimental studies and clinical applications in the highly dynamic field of functional restoration and repair in the central nervous system. This year, we will place the emphasis on new developments in Parkinson's disease, atypical Parkinsonian syndromes, gene therapy, and stem cells. In addition, there will be special lectures with comprehensive presentation on neuroprotection in Parkinson's and Huntington's disease, new therapies for stroke, and ethical issues when implementing new therapies for neurological disorders.

It is our pleasure to publish the abstracts of this meeting in this issue of the Zeitschrift "Regenerative Medizin". After organizing the NECTAR Meeting in 2006 and the International Neural Transplantation Meeting in 2008, we are welcoming you again in Freiburg and we thank all participants for their active contributions in this meeting.

PD Dr. med. Christian Winkler

LECTURES

Parkinson's disease

Value of current pharmacotherapy and deep brain stimulation

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Abstract not submitted

Future options for pharmacotherapy

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Abstract not submitted

Neural transplantation: current status in the TRANSEURO trial

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The ability to successfully use fetal dopaminergic cells derived from the developing human midbrain to restore the failing dopaminergic nigrostriatal pathway in Parkinson's disease (PD) has been achieved with clear benefits lasting for well over a decade in some cases. However, not all patients do equally well with this therapy and the reasons for this variability in outcome has become the subject of much debate, especially given that the transplants can induce side-effects such as graft induced dyskinesias. In this talk I will describe how the use of fetal ventral mesencephalic tissue transplants has evolved in the clinical arena over the last 20 years and what lessons we have learned and can learn from this. This will then form the basis for a discussion of how we can now move forward with reparative cell therapies for PD, and the shape of trails to come.

Dopamine-dependent dyskinesia in Parkinson's disease

News from imaging

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Parkinson's disease (PD) is associated with nigral degeneration and striatal dopamine deficiency. Demonstrating striatal dopamine terminal dysfunction with PET or SPECT supports the diagnosis of PD and helps to assess the long-term effects of dopaminergic medications. PET studies with ^{11}C -raclopride can be used to detect changes in striatal synaptic dopamine levels following pharmacological challenges and during specific motor or behavioral tasks. Using this technique it has been possible to demonstrate in vivo that in PD with L-DOPA-induced dyskinesias high levels of synaptic dopamine following acute administration of L-DOPA correlate with the severity of peak-dose dyskinesias and that continuous delivery of L-DOPA results in more stable dopamine levels and reduction of the dyskinesia. Furthermore, by using a specific ligand for the 5HT transporter (^{11}C -DASB) and PET we have

mapped serotonergic neuronal status in PD and explored the hypothesis that mishandling of L-DOPA from residual 5HT neurons in the striatum may contribute to the development of dyskinesias. Recently, with PET and ^{11}C -DASB we have also been able to demonstrate excessive serotonergic innervation in the grafted striatum of two patients with Parkinson's disease, who had exhibited major motor recovery after transplantation with dopamine-rich fetal mesencephalic tissue but had later developed off-medication dyskinesias. The dyskinesias were markedly attenuated by systemic administration of a serotonin 5-HT_{1A} receptor agonist, which dampens transmitter release from serotonergic neurons, indicating that the graft-induced dyskinesias were likely to be caused by the serotonergic hyperinnervation.

News on L-DOPA-induced dyskinesia I

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During the past 20 years, theories about the mechanisms of L-DOPA-induced dyskinesia have swung between pre- and post-synaptic sites within the nigrostriatal dopamine system, but an integrated understanding of this movement disorder has remained elusive. Functional imaging studies in human Parkinson's disease patients have uncovered an association between L-DOPA-induced dyskinesia and indexes of presynaptic dopamine dysregulation, such as the magnitude of striatal dopamine release following a standard L-DOPA dose. These studies have not, however, ruled out an involvement of post-synaptic mechanisms, such as an abnormal response of striatal neurons to the treatment. In our laboratory, we are taking advantage of a validated rat model of L-DOPA-induced dyskinesia in order to dissect the contribution of pre- and post-synaptic alterations to the development of this movement disorder. In this model, the nigrostriatal pathway is severed with unilateral injections of 6-OHDA, and chronic treatment with L-DOPA is used to induce abnormal involuntary movements that mimic peak-dose dyskinesia in Parkinson's disease. In behavioural pharmacological studies, we are now comparing and correlating the abnormal involuntary movement scores induced by either L-DOPA or a D1 receptor agonist in the same group of animals. We are also comparing the magnitude of the antidyskinetic effects induced by treatments that affect either dopamine release or post-synaptic responses. Via a post-synaptic mechanism, specific antagonists to metabotropic glutamate receptor 5 (mGluR5) produce antidyskinetic effects and block dyskinesia-associated alterations in striatal nuclear signaling. On the presynaptic level, we have discovered a new mechanism of maladaptive plasticity affecting striatal serotonin axon terminals, which constitute a source of dysregulated dopamine release following the administration of L-DOPA. Striatal serotonin fiber density is dose-dependently enhanced by L-DOPA treatment in the rat model of L-DOPA-induced dyskinesia. SERT autoradiography was performed on striatal tissue from rats, monkeys and humans showing elevated levels of serotonergic innervation in dyskinetic cases compared to non-dyskinetic ones. This presynaptic plasticity should be considered as a susceptibility factor for dyskinesia. Taken together, L-DOPA produces dysfunctional neuroplasticity affecting both pre- and post-synaptic compartments in the nigrostriatal system. Depending on the specific profile of the plastic changes occurring in the individual parkinsonian brain, a specific antidyskinetic treatment might be more or less appropriate in different subjects. Our findings will help to devise biomarkers for patient stratification and monitoring in clinical trials of antidyskinetic interventions.

The critical role of the pre-synaptic compartment in the induction and maintenance of L-DOPA-induced dyskinesia (News on L-DOPA-induced dyskinesia II)

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L-DOPA-induced dyskinesia (LID) is a major problem in management of patients with Parkinson's disease. The underlying mechanisms of LID, however, are still not fully understood. Current views suggest that changes in both pre-synaptic (i. e., production, storage, controlled release and re-uptake of dopamine (DA) by nigrostriatal DAergic neurons) and post-synaptic (i. e., status of receptors and second messenger signalling pathways) components are critical in induction and maintenance of dyskinesias. In experimental studies, the destruction of the pre-synaptic DA terminals by administration of a specific neurotoxin, and the plastic changes induced in the post-synaptic striatal neurons occur at the same time. Moreover, synaptic changes secondary to chronic drug treatment further complicate the interpretation of the observations. Therefore studying the individual contribution of each compartment using the conventional toxin models is not possible. To dissect out the individual contribution of the pre- and post-synaptic compartments in induction and maintenance of LIDs, we generated adeno-associated viral vectors coding for a shRNA to knockdown tyrosine hydroxylase expression, the rate-limiting enzyme in DA synthesis, to functionally silence DA production without affecting the structural integrity of the DA terminals. This approach allowed us to create a unique model to study the specific role of the pre-synaptic compartment in LID development in rats. TH knockdown in the nigrostriatal projection neurons led to 70% decrease in extracellular DA concentration in the striatum. To test if post-synaptic neurons would develop supersensitivity to direct DA receptor stimulation in these animals, we challenged the animals with a direct D1/D2 agonist. Animals with decreased DA production developed dyskinesias following chronic apomorphine stimulation. Interestingly, chronic treatment of L-DOPA failed to induce any abnormal movements in these animals suggesting that the induction of LIDs are controlled by the pre-synaptic compartment. We then challenged the apomorphine treated animals with a single high dose of L-DOPA to observe if the already established dysplastic changes can be activated with L-DOPA in the rats with functional DA depletion. These animals remained similar to both the intact and scrambled shRNA control animals during the entire observation period of 150 min after the drug treatment. Analysis of the immediate early genes provided further evidence that L-DOPA treatment does not induce abnormal activation of the post-synaptic neurons when the DA handling is intact. These data suggests that the pre-synaptic DA handling compartment is the critical determinant of both the induction and maintenance of the L-DOPA-induced dyskinesias.

News on graft-induced dyskinesia

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Transplantation of fetal dopamine (DA) neurons in patients with Parkinson's has been compromised by development of a new type of off-medication dyskinesia now known as graft-induced dyskinesia (GID). Various hypotheses have been discussed to be the cause of GID, such as inclusion of serotonin neurons within the graft suspension or grafting into hosts with severe preoperative L-DOPA-induced dyskinesia (LID; see also Lane et al, 2010, Prog Brain Res 184: 295-309). In our recent studies we have therefore analyzed the expression of LID and GID with respect to different proportions of DA and serotonin neurons within a graft, or with respect to the existence of pre-operative LID.

Rats with complete unilateral 6-hydroxydopamine-induced lesions of the nigrostriatal system received intrastriatal mesencephalic grafts containing different proportions of DA and serotonin neurons (ratio TH/5HT from 1:1 to 1:10). All grafted groups showed functional recovery seen in amphetamine-induced rotation and animals with larger DA grafts also showed improvement in spontaneous forelimb use in the cylinder test. LID was reduced in all groups containing DA neurons, and this reduction was more pronounced in groups with a higher proportion of DA neurons (approx. 80% reduction) as compared to the groups containing less DA neurons. Even animals with a high number of serotonin neurons as compared to DA neurons in the striatal grafts (ratio TH/5HT up to 1:10) did not show an increase of pre-operative LID. Thus, the negative effects of serotonin neurons on LID were prevented even by small numbers of DA neurons in the graft. GID was observed in all grafted groups and was more severe in animals with larger DA grafts as compared to those with low TH/5HT ratio suggesting that DA but not 5-HT neurons are responsible for development of GID. Interestingly, both the reduction of LID and the induction of GID were related to the graft-induced DA fiber density in the caudolateral striatum, suggesting that good behavioral effects or LID-reduction may be accompanied by a risk for the induction of GID. Nonetheless, GID was mild in all animals except for one. Overall, serotonin neurons did not play a role for the induction of GID in our studies and even small numbers of DA neurons prevented a negative effect of serotonin neurons on LID. We then performed intrastriatal grafting of regular DA grafts in animals with either very mild or more severe preoperative LID. These grafts were effective in improving complex motor behavior in both groups, and effectively reduced LID. In animals with more severe preoperative LID, GID was observed in some of the animals, while GID did not appear or was very mild in animals with mild preoperative LID. This suggests that patients with severe LID are less good candidates for grafting.

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Gene therapy in Parkinson's disease

Optimized rAAV5-mediated continuous DOPA delivery as a potential therapy for Parkinson's disease

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It has been hypothesized that complications related to oral administration of L-DOPA in Parkinson's disease (PD) may, at least in part, be due to the intermittent, pulsatile supply of L-DOPA provided by peripheral administration. Thus, we are currently developing a novel gene therapy strategy to provide long-term continuous DOPA administration using recombinant adeno-associated viral vector serotype 5 (rAAV5) to over-express tyrosine hydroxylase (TH) and GTP-cyclohydrolase (GCH1) enzymes. In a number of studies, we have determined not only the optimal stoichiometric relationship between TH and GCH1 genes for ectopic DOPA production and the cellular machinery involved in its synthesis, storage and metabolism but also the kinetics and magnitude of motor recovery achieved using this mode of dopamine replacement. Taken together, these results strengthen the evidence that this gene therapy strategy can be utilized as a competitive treatment alternative for PD patients, especially those in the complication phase.

Safety and efficacy of enzyme dopamine gene therapy in Parkinson's disease (PD): a phase I clinical trial

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Oral dopaminergic treatments remain the primary standard of care for PD. Although highly efficacious in the early stages of disease, their intermittent intake is associated with debilitating long term side effects in more advanced disease that seriously impact on the quality of life and restrict their long term use. We have developed a lentiviral vector (ProSavin[®]) derived from the equine infectious anaemia virus expressing the three key dopamine biosynthetic enzymes (tyrosine hydroxylase, aromatic L-amino acid decarboxylase and GTP cyclohydrolase-1). ProSavin has been demonstrated to mediate dopamine production and cause behavioural correction in the rat (Azzouz et al., 2002) and in a severe MPTP-lesioned non human primate model (Jarraya et al., 2009). In a phase I, open label clinical study six PD patients have been evaluated at two doses of ProSavin (group 1, 1x, n=3, group 2, 2x, n=3). All patients have completed at least 12 months follow up. The primary endpoints of the study are: (1) the number and severity of any adverse events associated with ProSavin administration, including the incidence of dyskinesias, and (2) the patients' motor responses to ProSavin administration by assessment of UPDRS Part III, six months following surgery. Overall, ProSavin was safe and well tolerated. There were no "OFF" state dyskinesias, no immune responses to ProSavin and no surgical or any other serious adverse events. The average improvement in motor function at one year (UPDRS III "OFF" score) was 28% relative to the patients' pre treatment motor function.

Special Lecture 1

Trophic factor gene therapy for Parkinson's disease and Huntington's disease: the path to the clinic

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The gene delivery of trophic factors has long been proposed as a novel therapeutic strategy for the treatment of neurodegenerative diseases. This presentation will review preclinical studies performed in multiple nonhuman primate models of Parkinson's disease. These studies demonstrate the safety, tolerability, and efficacy of gene delivery of the trophic factor neurturin in these model systems. These preclinical studies have lead to phase I and phase II clinical trials which will be reviewed and the rationale and study design of a new ongoing Phase I/II trial will be discussed. We have also demonstrated that this same trophic factor is safe and well tolerated in multiple rodent models of Huntington's disease. This lecture will review these studies including the important observation that intrastriatal AAV delivery of neurturin is not only well tolerated and preserves motor function, it can protect both striatal and cortical neurons in a transgenic mouse model of Huntington's disease. A planned Phase I clinical trial using gene delivery of neurturin in patients with Huntington's disease will also be discussed.

Huntington's disease

What is needed vs. what would be interesting to know in stem cell therapy for Huntington's disease

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Abstract not submitted

Neural transplantation in Huntington's disease

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Replacement of striatal projection neurons by transplantation of fetal whole ganglionic eminence (WGE)-derived cell suspensions is an attractive therapeutical approach for the treatment of Huntington's disease (HD). The ongoing European multicentre trial MIG-HD currently investigates beneficial effects of neurotransplantation in HD patients. We here present available data from the German branch of the trial on safety, side effects and functional parameters. Risk assessment is based on pharmacological safety, involving immune rejection, tumour formation, disease transmission and side effects of immune suppression. Functional assessment involves motor scoring and cognitive tests. Patients enrolled in the trial received fetal WGE grafts. Tissue was processed mechanically and injected by stereotactic implantation into up to six trajectories of the striatum bilaterally. Tissue was obtained from elective abortions. Blood samples from women undergoing abortions were analyzed for viral infections. Testing for bac-

terial contamination was performed after implantation. 15 HD patients were transplanted bilaterally and 1 unilaterally, receiving tissue from 1–2 donors/side. Age at enrolment ranged from 31 to 53 years. No transmission for HIV, HBV, HCV or HTLV. Fetal neurotransplantation was not associated with severe side effects in most patients. In a single patient, a bacterial cerebral infection (*Staphylococcus* sp.) was detected closely after surgery. One patient who had suffered from a chronic depression committed suicide and the brain could be evaluated by immunohistochemistry. The most common side effects were associated with immune suppression. There was no case of graft overgrowth. One patient deteriorated shortly after withdrawal of immune suppression and improved after administration of high-dosed steroids. The time course of clinical changes after transplantation suggests that during the initial 6 months after transplantation, factors like the surgery or the onset of immune suppression result in a slight deterioration. Although most patients had decreasing functional benefits during the longterm course, a substantial number of patients improved or stabilized after a variable interval following surgery. Clinical whole ganglionic eminence cell transplantation in HD has been shown to be generally safe and feasible. The potential immunogenicity of the grafts and the ongoing degeneration of the host brain might inhibit more substantial clinical beneficial effects in the longterm course of the disease. Our data indicate general safety and promote further refinements of the method and upcoming trials using alternative cell resources in HD.

Pallidal deep brain stimulation (DBS) in Chorea Huntington: preliminary results of a phase I trial

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Introduction: At present, there is no effective treatment or cure for Huntington's disease (HD) patients. Therefore, neural stem cell transplantation seemed to offer a potential treatment for HD patients that may slow down this devastating illness. However there remain major concerns in transplantation. Therefore our group looked for alternatives, utilizing deep brain stimulation (DBS), based on the long-lasting successful treatment of other neurodegenerative movement disorders like Parkinson's disease (PD). Questions remained concerning the optimal target. **Methods:** This phase I clinical trial is based on the hypothesis that deep brain stimulation of the internal pallidum can reduce choreatic symptoms in 6 HD patients. In addition, this trial should demonstrate which target point within the pallidum can be used effectively for specific features of HD in order to further refine this promising strategy for a phase II multicenter trial approach. We report on six consecutive cases who underwent DBS of the pallidum (GPi/GPe region). Electrodes were stereotactically implanted under general anaesthesia, followed by the implantation of a neurostimulation system (Kinetra, Medtronic). Patients were randomized to be stimulated into the GPi or GPe and cross over, each for 6 weeks. Then best contact stimulation was applied for 3 months. **Results:** No perioperative complications occurred. The coordinates for the active contacts in the GPi/GPe range were adapted to individual anatomical variability. Under DBS of the pallidum choreatic movements could be reduced by median of 70% in UHDRS (range 50 to 80). The quality of life (ADL) was significantly improved in five patients. Since the effects are delayed, the adjustment and testing of the remaining contacts took place in the course of 6 months postoperatively. The most effective

active contacts were in projection of the border of GPi and GPe. **Conclusion:** Systematic positive influence of DBS in Huntington's disease patients is reported for the first time. In the context of the following study it will have to be clarified whether the internal or the external part or other targets are suitable for DBS and which long-term results can be obtained.

News from other neurological diseases

Multiple system atrophy: current status in experimental and clinical studies

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Multiple system atrophy (MSA) is a sporadic and rapidly progressive neurodegenerative disorder presenting with autonomic failure in combination with parkinsonism or cerebellar ataxia. Over the last five years, significant progress has been achieved in understanding the pathogenesis of the disease. For example, important insights into the genetics of MSA have confirmed the key pathogenic role of α -synuclein. Advances in the early recognition of MSA have resulted in revised diagnostic criteria including for the first time neuroimaging indices. Finally, novel therapeutic options targeting disease modification have been evaluated in clinical trials. These include riluzole, recombinant human growth hormone and minocycline. Although the trials were negative, they generated important trial expertise in MSA and were only possible because of the establishment of international networks such as EMSA (www.emsa-sg.org) or its North-American counterpart NAMSAs. Currently, rasagiline is assessed in a multicentre phase II trial and results are expected by the end of 2011. Neurorestorative strategies have received less attention. However, there is some experimental evidence that it may be possible to improve a suboptimal levodopa response in parkinsonian MSA patients by embryonic striatal grafts. Further work is needed to characterize the effects of underlying alpha synucleinopathy on striatal graft function in transgenic MSA models.

Progressive supranuclear palsy: current status in experimental and clinical studies

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Progressive supranuclear palsy (PSP, Steele-Richardson-Olszewski syndrome) is a sporadic and progressive neurodegenerative disease with an average prevalence of 5.3 per 100 000. The mean age at onset is approximately 63 years. The classical clinical picture of PSP is characterized by a symmetric, akinetic-rigid syndrome, vertical supranuclear gaze palsy, frontal deficits, prominent postural instability and falls, and seems to comprise about half of the patients. However, several variants in the clinical presentation of pathologically confirmed PSP have been described recently, complicating the early differential diagnosis. To date there is no effective symptomatic, disease-modifying or neuroprotective therapy available. As a first step towards the development of an effective therapy, a detailed understanding of the disease mechanisms is required. PSP belongs to the family of tauopathies and involves both cortical and subcortical structures. Both neuropatho-

logical and human genetic observations and experimental results from transgenic models point to a central role of dysfunction or dysregulation of the tau protein in the pathophysiology of PSP. However, the way and sequence in which the putative aetiologic factors cooperate to mediate increased levels of the 4R-tau isoform, abnormal tau hyperphosphorylation, formation of neurofibrillary tangles and ultimately cell death remain unknown. Recent studies provided evidence for mitochondrial dysfunction in PSP. There is also a strong genetic component in the aetiology of PSP, since some rare cases follow an autosomal dominant mode of inheritance, and sporadic patients with PSP have more first-degree relatives with parkinsonism compared to controls. Recently gained insights into the pathophysiology of this disease have led to several hypothesis-driven therapeutic approaches aiming at disease-modification rather than mere symptomatic neurotransmitter replacement therapy. Agents targeting mitochondrial dysfunction have already shown a positive effect in a phase II study and further studies to verify and expand these results are ongoing. Clinical studies with agents targeting tau dysfunction such as tau-kinase inhibitors, tau-aggregation inhibitors and microtubule stabilizers are in preparation or ongoing. Studies are underway to further define the precise identity of the genetic and environmental factors and their interplay to trigger the PSP-specific sequence of pathological events. Present knowledge has already facilitated the implementation of a series of rational clinical studies. These studies are likely to reveal whether interference with the identified mechanisms will result in the development of urgently needed disease-modifying therapies.

Spinal cord injury: current status in experimental and clinical studies

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Over the last 3 decades, there has been substantial progress in identifying mechanisms that contribute to the limited recovery after spinal cord injury. Factors extrinsic to injured neurons and their axons such as inhibitory extracellular matrix, myelin-based inhibitors, chemorepellents, inflammatory responses and a lack of growth stimulating molecules contribute to the limited plasticity and lack of axonal regeneration. In addition, insufficient activation of cell-intrinsic regeneration programs, oligodendroglial cell death and poor remyelination limit behavioural improvements after spinal cord injury. The complexity of these mechanisms presents an enormous challenge for the development of therapies, and treatments that target a single factor may not be sufficient to induce robust changes in outcomes. Despite these obstacles, several strategies have been reported to generate functional recovery in animal models of spinal cord injury and have progressed to clinical trials. We will review some of the current strategies and the difficulties encountered when translating therapeutic approaches from animal models of spinal cord injury to patients.

Special Lecture 2

Ethics and societal aspects in transplantation and gene therapy approaches for neurological diseases

Gottweis H

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Transplantation in the central nervous system and gene therapy pose a set of interrelated societal and ethical challenges. Issues of social perception and normative preferences intersect with ethical reasoning and decision-making. The presentation will map the interaction of society, ethics, CNS transplantation and gene therapy, identify critical and controversial issues of today and in the future, and make suggestions for how to deal with these issues in a manner that satisfies ethical reflection and societal preferences and attitudes. Special attention will also be given to how to deal with the absence of knowledge in the public about cell transplantation and gene therapy in a constellation where these approaches gain importance in the clinical context.

Stem Cells

Neural stem cells

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Human neural stem cells may serve as a virtually unlimited source of specialized cells for transplantation in neurodegenerative diseases like Parkinson's disease. Neuronal differentiation of human stem cells has been achieved using a variety of strategies. However, production of dopaminergic neurons from neural stem cells isolated from post-mortem fetal brain tissue has proven difficult. We have investigated the effect of the anti-apoptotic protein Bcl-xL and oxygen tension on dopaminergic differentiation and survival of a human ventral mesencephalic stem cell line (hVM1). hVM1 cells and a Bcl-xL overexpressing subline (hVMbcl-xL) were differentiated by sequential treatment with fibroblast growth factor-8, sonic hedgehog, forskolin and glial cell line-derived neurotrophic factor. After 10 days at 20% oxygen, hVMbcl-xL cultures contained significantly more tyrosine hydroxylase (TH)-positive cells than hVM1 control cultures. This difference increased significantly from 11 ± 0.9% to 17 ± 0.2% of total cells when the oxygen level was lowered to 3%. Immunocytochemistry and q-PCR analysis revealed expression of several dopaminergic markers besides TH, just as dopamine was detected in the conditioned culture medium by HPLC analysis. Regarding other effects of Bcl-xL overexpression, it was confirmed to reduce cell death in the cultures. It did not change the relative content of GABAergic neurons but reduced the content of astroglial cells compared to control. We conclude that Bcl-xL and lowered oxygen tension act in concert to enhance dopaminergic differentiation and survival of human neural stem cells. This issue will be discussed along with our recent strategies and experience in generating TH-positive neurons from human neural stem cell lines.

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Neural lineage specification from human pluripotent stem cells

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Embryonic and other pluripotent stem cells represent an appealing cell source for biomedical studies and experimental therapies aimed at alleviating neurological disease. However, the processes that control growth and directed differentiation in vitro and after transplantation have remained poorly understood. In this talk, we present recent progress in the derivation and study of human embryonic stem (ES) and induced pluripotent stem (iPS) cell-derived neural cells, discussing induction protocols, cell sorting approaches and transplantation. We highlight key challenges in the generation of neural cell types for biomedical applications, and outline how ongoing work aimed at elucidating the mechanisms of cell interaction via diffusible modulators and via surface molecules may contribute to developing better-suited neural phenotypes for future cell therapeutic options.

Microglia differentiation and microglia replacement strategies in neurodegenerative diseases

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Introduction: Microglia are the phagocytes of the brain. They have been implicated in the underlying causes of several degenerative diseases including Alzheimer's and Huntington's. They display senescence, a loss of function and loss of balanced regulation during aging. Neuronal stem cell transplantation and mesenchymal stem cell transplantation have shown some beneficial results in neurodegenerative disease models. The role of microglia in regenerative processes and as a basis for cell replacement therapies are unclear. Aim: We wanted to demonstrate function and high yield of microglia differentiated from adult stem cells and from human induced pluripotent stem cells (iPS). In transplantations their ability to invade brain tissue, and effects on microglia activation and neurogenesis were studied. Methods: Bone marrow stem cells and human IPS were differentiated to microglia employing various combinations of selective adhesion, astrocyte conditioned medium (ACM), Flt3L, SCF and GM-CSF supplementation. Uptake of fluorescent beads, burst in ROS production and microglia markers (CD11b/CD45, F4/80) were quantified using flow cytometry. Transplanted cells were tracked by sex mismatch. For microglia activation and neurogenesis, Iba-1, MHCII, DCX and Ki-67 were used as markers for stereology based cell counting. Regulation of inflammation and neurogenesis was measured with PCR arrays. Results: The cells differentiated from adult bone marrow stem cells show function (phagocytosis, oxidative burst) and markers (CD11b+/CD45+, F4/80+) of primary microglia. Human induced pluripotent stem cells could also be shown to differentiate into microglia like cells and were positive for microglia marker. Transplanted cells were found in bone marrow of single mice but not in telencephalon, liver or lung. Transplantation of stem cells modified the inflammation level and neurogenesis in the mice. Conclusion: We demonstrate functional ca-

capacity of microglia differentiated from non adherent bone marrow cells and show the first steps toward an establishment of IPS differentiation. Transplanted progenitors migrate at least in part to the bone marrow and show some functional improvement in aged and Alzheimer mice.

Stem cells for Parkinson's disease

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The widespread implementation of cell replacement therapies is dependent on a readily available and plentiful source of cells that are both safe and efficacious. Human embryonic stem (hES) cells potentially represent such a cell source, however issues such as poor efficiency of neuronal differentiation and overgrowth formation in vivo has limited their translation to the clinic. Using a novel protocol for floor plate induction in vitro (dual inhibition of SMAD signalling; Fasanò et al. 2010) we seek to recapitulate key events during development in vitro and pattern the cells to a midbrain identity, with the final aim to increase the yield of transplantable mesencephalic dopaminergic neural progenitors from hES cells. We have assessed transplants of hES that have been extrinsically patterned in vitro to form floor plate-like cells. The cells were transplanted into the striatum of adult 6-hydroxydopamine-lesioned rats. The rats were immunosuppressed using daily injections of cyclosporin A for the extent of the study. After at least 4 weeks post-transplantation the animals were perfused for histological analysis to assess the maturity of the grafts and their ability to form mature mesencephalic dopamine neurons, and also to monitor the occurrence of overgrowths. After 4 weeks, a large number of FOXA2+ cells survive transplantation without incidence of overgrowths, or obvious proliferation in the transplants. At this time point only few cells express TH. Ongoing studies are aimed at increasing the yield of dopamine neurons in vitro and in vivo by expressing key dopamine determinants and/or supplying extrinsic patterning agents.

Clinical applications of adherent human neural stem cells

Johe K

Neuralstem, Inc., Rockville, MD, USA

Neuralstem, Inc. is a small biotechnology company founded in 1996 in Maryland, USA, to develop adherent human neural stem cells toward clinical applications. I will summarize briefly the nature of the cells and current status of clinical development.

Special Lecture 3

Stroke: current status in experimental and clinical studies

Kokaia Z

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In acute neurodegenerative disorders following a sudden insult neurons are rapidly damaged and usually die but cellular loss can occur hours and days thereafter. These diseases cause massive morbidity and mortality and tremendous economic and societal burden, especially ischemic stroke, which is a leading cause of death and disability in adult humans. In recent years, this recovery phase of stroke attracted

much of the attention of researchers and clinicians, and currently is considered as most suitable target for the stroke therapy. This is justified by the long-term therapeutic window and also intrinsic plasticity-based mechanism of recovery which is operating in the brain and represents suitable target of the therapy. These therapeutic approaches would be initiated many hours, if not days and weeks after stroke onset, with the intention of improving neurological function and not necessarily reducing the burden of the ischemic lesion. The aim of such therapeutic strategy is to enhance and accelerate the spontaneously operating self-repair/recovery mechanism. Stem cells have the capacity to generate neurons and glia cells which are lost in neurodegenerative diseases including stroke. The adult brain's own neural stem cells are potential novel therapeutic targets because they produce neurons and glia in response to injury and could become affected by the degenerative process. Besides cell replacement, stem cell-based approaches can also improve function by modulating inflammation, preventing neurons from dying, and increasing angiogenesis. These exciting laboratory findings should now be responsibly translated to the clinic. Some initial studies using cell therapy approaches have been performed in patients with stroke. There are several ongoing clinical trials in stroke patients, mainly using autologous bone-marrow-derived cells. However, many issues remain before stem cell therapy can advance to full-scale clinical trials. These issues are (i) type of cells suitable for transplantation and their mechanisms of action, (ii) how to control proliferation, survival, migration, differentiation and integration of endogenous and grafted stem cells in stroke-damaged brains, and (iii) procedures for cell delivery, scaling-up, optimum functional recovery, and patient selection and assessment.

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DATABLITZ SESSIONS

Bone marrow-derived mesenchymal stem cells are recruited to the brain but do not differentiate into neuronal phenotypes in mouse models of stroke

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Recent studies have highlighted the possibility that endogenous bone marrow-derived stem cells (BM-MSCs) have the potential to give rise to cells of the neural lineage in the mammalian brain. However, it is not known whether this type of endogenous repair might contribute to production of new neurons after ischemic stroke. In the present study, we therefore investigated the neurogenic potential of BM-MSCs in chimeric mice that underwent whole body irradiation followed by BM reconstitution with green fluorescent protein (GFP) expressing

transgenic BM-MSCs. Recruitment and differentiation of GFP-expressing cells was addressed in uninjured controls and in two different murine stroke models, the permanent distal middle cerebral artery occlusion (dMCAO) model and the hypoxia-ischemia (HI) model, which allows reperfusion of the ischemic parenchyma after induction of the insult. Mice were sacrificed at 4 weeks or 3 months after stroke, respectively, and the brains were processed for immunohistochemistry. Co-localization studies were carried out with GFP and the neuronal markers DCX, NeuN and Tuj1, the astrocyte marker GFAP, the oligodendrocyte progenitor marker NG2, the macrophage/microglia marker Iba1 and the endothelial cell marker vWF. In both the dMCAO and the HI model, we found engraftment of GFP-expressing BM-MSCs, particularly in the stroke borderzone, at 4 weeks and 3 months after the insult. GFP-positive BM-MSCs-derived cells did not co-localize with DCX, NeuN, Tuj1, or NG2. However, abundant co-localization was present with Iba1- and vWF-immunoreactive cells, and sparsely, GFAP-positive astrocytes also co-expressed GFP. The number of GFP-positive cells in the brain as well as the co-localization with Iba1 and vWF were significantly higher in mice after dMCAO or HI when compared to non-stroked controls. Therefore, our data suggest that endogenous BM-MSCs in the systemic circulation are recruited to the post-ischemic murine brain, but do not contribute to the generation of new neurons. On the other hand, our findings further support the importance of microglia and endothelial progenitors derived from BM-MSCs for the inflammatory response and angiogenesis after ischemic stroke.

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Creatine improves the metabolic state of murine and human neural stem cells and improves expansion and neuronal induction

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The creatine kinase (CK)/phosphocreatine (PCr) system plays a central role in cellular energy buffering and energy transport, particularly in cells with high and fluctuating energy demands like neurons. Given the high expression of CKs in the developing central nervous system, Cr supplementation might improve the metabolic state of neural stem cells (NSCs). In the present study, we investigated the effects of Cr on metabolic parameters, expansion, differentiation, and migratory capability of NSCs isolated from the subventricular zone of postnatal mice (mNSCs) and NSCs derived from the human fetal cortex (hNSCs). We found both the brain-specific cytosolic (BB-CK) and the ubiquitous mitochondrial (uMt-CK) isoform of CK expressed in mNSC and hNSC. Accordingly, Cr supplementation at 5 mM for 7 days resulted in higher CK-specific activity as well as PCr and ATP levels in mNSCs and hNSCs, as compared to untreated controls ($p < 0.01$). In both mNSCs and hNSCs, Cr exposure during expansion resulted in a dose-dependent increase in neurosphere size and total cell numbers with a maximal effect at 5 mM after 7 days in vitro ($p < 0.05$). Analysis of

BrdU incorporation did not reveal a significant increase in proliferation activity, but Cr-treated cultures contained less cells with immunoreactivity for active Caspase-3 ($p < 0.05$), pointing towards an anti-apoptotic effect of Cr. Using a modified Boyden chamber assay, we found that both Cr-exposed mNSCs and hNSCs demonstrated an improved migratory potential ($p < 0.01$) after 60 min, as compared to untreated controls. In line with this finding, we found that Cr-pretreated, GFP-labelled mNSCs showed improved migration to the ischemic brain area after intrastriatal transplantation in a distal MCA occlusion stroke model in C57/Bl6 mice ($p < 0.05$). Finally, Cr supplementation resulted in higher neuronal cell numbers (TuJ1+, $p < 0.05$) at the expense of the glial fate (GFAP+, $p < 0.05$) after differentiation in vitro for 5 days. In addition, we found disproportionally higher numbers of GABA-immunoreactive cells in Cr-treated cultures ($p < 0.05$), suggesting that Cr acts as a differentiation factor for specific neuronal subpopulations. In sum, our findings suggest that the CK/PCr system is critically involved in maintaining the energy metabolism of mNSCs and hNSCs. Chronic Cr supplementation resulted in increased CK activity, improved cellular ATP and PCr levels, inhibited apoptosis during in vitro expansion, and promoted NSC migration in vitro and in vivo. Cr exposure also promoted the differentiation of NSCs towards the neuronal lineage, particularly supporting the GABAergic phenotype. Cr pretreatment of NSCs might therefore offer new ways for improving cell replacement approaches for stroke and other diseases of the nervous system.

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FGF-2 modulates the late stages in embryonic development of the nigral dopaminergic neurons in mice

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The endogenous fibroblast growth factor 2 (FGF-2) is a physiologically relevant neurotrophic factor in the developing and adult nigrostriatal system (Grothe and Timmer 2007, Brain Res. Rev.). Our previous studies revealed additionally to a protection function of FGF-2 in mature mesencephalic dopaminergic (mDA) neurons an involvement of FGF signalling in developing substantia nigra pars compacta (SNpc). The increased numbers of tyrosine hydroxylase immunoresponsive (THir) neurons in SNpc of adult FGF-2 deficient mice, as well as decreased numbers in FGF-2 overexpressing mice suggest a regulatory role of FGF-2 for proper development of SNpc (Timmer et al., J. Neurosci., 2007). To elucidate the physiological function of FGF-2 in the nigrostriatal development, we analyzed embryonic (E14.5), newborn (P0), and juvenile (P28) FGF-2 deficient mice. Our recent stereological analysis (CASTgrid, Olympus) on the content of THir cells in the SNpc of FGF-2 depleted mice could further delineate the potential onset of the phenotype between E14.5 and P0. Additionally, the examination of the SNpc and ventral tegmental area (VTA) in juvenile mice (P28) showed a specific increase in number of THir cells in SNpc. The unchanged number of THir cells in VTA is suggestive for an unaltered migration of mDA neurons of FGF-2 deficient mice. To unravel the underlying mechanism, immunohistochemical analysis of proliferation rates in E14.5 animals and of apoptosis rates in P0 ani-

mals was performed. The significant increase in Ki67ir cells in the subventricular zone of FGF-2 deficient mice points out a modulating influence of FGF-2 on proliferative progenitors in ventral mesencephalon. The number of cells positive for cleaved caspase-3 was unchanged in the P0 animals, whereas the number of cells positive for apoptotic bodies but negative for cleaved caspase-3 was significantly decreased in the mutants. This might indicate either a later onset of natural cell death or reduction of caspase independent cell death. The examination of the expression levels of mDA relevant genes by qRT-PCR in E14.5 and P0 did not reveal a possible target modulated by FGF-2. However, both physiological alterations could determine the phenotype in the ventral mesencephalon of the adult FGF-2 depleted mice: longer or enhanced proliferation of progenitors may cause an increased generation of mDA neurons, while altered wiring control during the maturation may lead to reduced natural cell death. Moreover, the impact of signalling pathways activated downstream of FGF receptor will be investigated in the recently established in vitro model of nigrostriatal co-culture explants.

Two-step grafting – a new method to enhance cell survival and study graft development in a 6-OHDA rat model of Parkinson's disease (PD)

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Background/Aims: Transplantation studies in the 6-OHDA rat model of PD have shown that only 8–10% of the grafted embryonic ventral mesencephalic (VM) dopaminergic (DA) cells survive. In order to increase this rate, we established a new two-step grafting protocol where a standard amount of cells is divided in half and grafted in 2 separate sessions with a defined time interval. We furthermore give an insight into the underlying mechanisms by altering the time intervals, the amount of cells in the 1st graft and by evaluating the 2 grafts independently using GFP+ cells. Methods: 48 6-OHDA-lesioned rats were divided into 3 two-step grafting groups, each with a time interim of 2, 5 or 9 days between the 2 transplantation sessions. Each group was subdivided in 2 sub-groups receiving either 200000 (low cell number groups: 2dL, 5dL, 9dL) or 400000 (high cell number groups: 2dH, 5dH, 9dH) GFP-VM cells in the 1st grafting session. For the 2nd transplantation, all groups received the equal amount of 200000 GFP+ cells. As control, 2 standard transplantation groups (standard low/high cell number groups, StL/StH) were grafted with the same constellation of cells in a single operation session. Transplantation effects were evaluated by drug-induced rotation tests 2 and 6 weeks after the 1st grafting. The animals were sacrificed 8 weeks after transplantation and graft survival was evaluated by stereology. Results: In the rotation test all transplanted groups showed significant compensation from the lesion. Morphological analysis showed a better survival of DA cells in the 2d groups in comparison to all other transplantation groups. The cell number in the 2dH group was significantly higher than in the 5dH (+ 50%), the 9dH (+ 68%) and the StH standard group (+ 47%). The 2dL group exhibited an even higher increase of + 137% as compared to the StL group. Furthermore, the transplants in the 2d groups had higher graft volume and DA-fibre density values as compared to all other two-step grafting groups. In addition, as an incidental finding, some grafts showed intense growth of GFP+ (2nd graft derived) vessels present in 7/8 of the 2dH, 4/8 of the 2dL, 5/7 of

the 5dH, 2/7 of the 5dL, 1/6 of the 9dH and 3/7 of the 9dL transplants. These vessel networks only formed in regions where both grafts overlap. Conclusion: Our findings show that two-step grafting with a 2 days time interval significantly increases DA cell survival in comparison to the standard protocol. As a potential mechanism we found an increased donor-derived vessel formation which only occurs under specific conditions.

Plasmid-based, small molecule augmented reprogramming of human fibroblast cultures into a pluripotent state and subsequent neural induction

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Reprogramming human somatic cells into a pluripotent state and subsequent differentiation into the desired cell type offers a potential new source for replacement strategies, disease modelling or drug testing on patient-derived cells. Most protocols today employ viral vectors inserting into the genome for factor expression, however there have been reports of reprogramming achieved by plasmid vectors. In our study we used a linearized polycistronic expression plasmid carrying the sequence of the four Yamanaka factors (Klf4, Sox2, Oct4, c-myc) under control of the CAG promoter. Fetal and adult human fibroblasts were transfected by electroporation and transfection efficiency was estimated by expression of a mOrange reporter gene. Transfection efficiency tended to be very low (5 % max.), presumably due to the large size of the expression plasmid (ca. 13 kB). Transfected cells showed no reprogramming under pluripotent culture conditions. Concerted addition of a combination of small molecules (valproic acid, ascorbic acid, sodium butyrate, SB431542, PD035291) resulted in few cases in the formation of colonies resembling embryonic stem cells after two to three weeks that could be passaged on gamma-irradiated fibroblast feeder cells. Those colonies showed characteristics of pluripotent stem cells (alkaline phosphatase positivity, expression of SSEA-4, NANOG and TRA-1-80) and spontaneously differentiated into cells of all three germ layers in the embryoid body assay.

When treated following a protocol for neural induction under adherent conditions (Chambers et al.) those cells formed neural rosettes and showed PAX6 and Tuj-1 expression. By employing the plasmid-based reprogramming we could demonstrate the induced pluripotency could only be achieved by small molecule treatment in some cases. Once reprogramming was achieved, cells behaved very much like bona fide pluripotent stem cells. The most important drawback of this method stays however the low reprogramming efficiency, most likely due to the low transfection efficiency. These hurdles have to be overcome if the method should serve as an alternative for virus-based reprogramming.

Systemic administration of Neuregulin-1 β 1 rescues dopaminergic neurons in substantia nigra against 6-OHDA intoxication

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Neuregulin-1 (NRG1) is a growth and differentiation factor, which plays a crucial role in development and plasticity of the nervous system. The factor has genetically been linked to schizophrenia, a disease caused by imbalance in the development of the dopaminergic system. It is shown that human as well as rodent midbrain dopaminergic neurons abundantly express the ErbB4 receptor, which transduces the NRG1 signaling. Recently, it was demonstrated that injections of NRG1 in neonatal rodents increase the enzymatic activity of tyrosine hydroxylase, the rate-limiting enzyme in the dopamine synthesis, and induce a persistent hyper-dopaminergic state. Interestingly bioactive truncated forms of NRG1 β 1, including the soluble extracellular domain of the protein, have been reported to cross the blood-brain barrier in neonatal and adult rodents. Altogether these facts strongly suggest NRG1 β 1 as a good candidate for neuroprotection and possible symptomatic treatment for Parkinson's disease (PD), a neurodegenerative disorder characterized by progressive cell death of the midbrain dopaminergic neurons. In the present study, we have evaluated the neuroprotective effect on the dopaminergic system in mice after systemic (intraperitoneal) injections of a truncated form of NRG1 β 1. For this purpose we used the well-established 6-OHDA-induced toxicity mouse PD model. First, all animals received an intrastriatal 6-OHDA injection in order to achieve a partial lesion of the nigrostriatal dopamine system. The animals were then treated with daily low doses of NRG1 β 1 (50 ng/kg), for 8 days starting either 6 or 48 hours after the 6-OHDA injection. The animals were sacrificed at 28 days post 6-OHDA injection, i.e. 20 days after last NRG1 β 1 dose. We show here that the truncated NRG1 β 1 crosses the blood-brain-barrier, induces ErbB4-phosphorylation and increases nigral and striatal dopamine levels in healthy adult mice. Most important, however, we demonstrate that significant protection of dopamine fibers in the striatum and dopamine neurons in the midbrain against 6-OHDA-induced toxicity is achieved by systemic NRG1 β 1 administration.

In conclusion, we believe, in light of our and others' data, that that NRG1 β 1 may be a very promising candidate for neuroprotective, and also symptomatic, treatment for PD patients. However, further experimental and clinical investigations are strongly warranted.

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Multifunctionalized electrospun silk fibers provide a bio-active support for axonal growth in the central nervous system

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The repair of central nerves remains one of the major challenges in regenerative neurobiology. To create a regenerative guide possessing critical features such as cell adhesion, physical guiding and topical stimulation, we selected silk as the material and electrospinning as the technique. Fibroins from Bombyx Mori were chosen for their biocompatibility and nerve cell adhesion properties and electrospinning for its capability to create aligned biofunctional nanofibers. We demonstrated that silk can be used to obtain highly aligned electrospun nanofibers. Moreover, the addition of Brain Derived Neurotrophic Factor (BDNF), Ciliary Neurotrophic Factor (CNTF) or both factors to the fibers enabled them to be bioactive while it did not modify the structure or the surface of the fibers. Interestingly, only a very small fraction of the loaded growth factors were released over time. Entrapped factors remained active as rat retinal ganglion cells (RGCs) exhibited longer axonal growth when in contact with the biofunctionalized fibers. The growth of neurites was increased by 2 fold on fibers containing BDNF, 2.5 fold with fibers containing CNTF and by almost 3 fold on fibers containing both factors. Taken together, our results demonstrate the potential of aligned electrospun silk fibers to serve as a guide and as a reservoir for therapeutic molecules that need to be used topically. These observations are of great interest for the regeneration of the optic nerve but applications go far beyond the field of vision.

Fetal antigen-1 positive cell numbers are upregulated in the striatum of 6-OHDA lesioned rats

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Fetal antigen 1/delta-like (FA1/dlk) protein is a member of the epidermal growth factor superfamily. FA1/dlk has been proposed to be a growth and/or differentiation factor expressed in cells during development. Recently, others and we have demonstrated that FA1/dlk is expressed in the ventral mesencephalon. Colocalization experiments revealed that an extensive number of tyrosine hydroxylase (TH)-ir neurons also expressed FA1/dlk in the SNc assuming that these cells are projection neurons. This latter assumption was further substantiated by co-localization with retrogradely transported fluorogold. We have previously shown that a unilateral 6-OHDA lesion resulted in a marked loss of both FA1/dlk-ir and TH-ir neurons in the SNc. Accordingly, innervation of TH-ir and FA1/dlk-ir fibers were severely reduced in the lesioned striatum. Importantly, preliminary observations revealed an augmented number of striatal FA1/dlk-ir cells in the

denervated striatum. Hence the present study aimed at investigating in detail on the origin of these cells, i.e. whether the higher striatal number of FA1/dlk-ir cells in the lesioned side was the result of increased endogenous cell proliferation. For that purpose adult rats received bromodeoxyuridine (BrdU) injections for three days after an unilateral injection of 6-OHDA into the striatum. One week and one month after the lesions rats were perfusion fixed and the brains processed for histological analyzes. In line with our previous observation FA1/dlk-ir cell numbers were found to be upregulated in the striatum of 6-OHDA lesioned rats. Interestingly, this increase was only moderate after one week (by 25 %) and did not differ significantly from controls. In contrast, one month after the lesion significantly higher cell densities were detected (by 75 %). Our immunohistochemical analyzes revealed no colocalization of FA1/dlk-ir cells with BrdU. Similarly, the striatal FA1/dlk-ir cells were found not to express DCX and Ki67. In line with this notion we could demonstrate that FA1/dlk-ir cells expressed the neuronal marker NeuN and a subpopulation colocalized with DARPP-32. Moreover, no colocalization was observed with GFAP or OX-42. These observations hint to the view that FA1/dlk-ir cells were not newly generated but rather that FA1/dlk was up-regulated in already existing neurons in response to the lesions. In conclusion, our findings show that FA1/dlk expression is dynamically regulated in response to lesions of the nigrostriatal system and further support the view that FA1/dlk may play an important role in Parkinson's disease.

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Regulation of endogenous GAD67 expression using artificial transcription factors

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The loss of dopaminergic input from the substantia nigra pars compacta to the striatum seen in Parkinson's disease (PD) has previously been shown to give an altered level of GAD67 and release of GABA from the striatum. GAD67 is a rate-limiting enzyme in the synthesis of GABA and by modifying the amount of GAD67, the GABA output may be altered. We have constructed artificial transcription factors (ATFs) that target specific sequences in the GAD67 promoter. They currently contain a VP64 activating domain to mediate up-regulation of the gene. The ATFs were evaluated in vitro and in vivo using lentiviral vectors, with the ATF under the control of a CMV promoter. In vitro, PC12 cells were infected at MOI 0.5, 1 and 5. The level of GAD67 protein was studied using Western blot and the toxicity of the ATFs was studied using a MTT cell proliferation assay. Two ATFs were evaluated: G1 and G3. The ATF G1 did not show any significant up-regulation of GAD67 compared to uninfected PC12 cells and cells infected with a gene encoding GFP. On the other hand, expression of the ATF G3 showed a 9-fold up-regulation of GAD67 protein at an MOI of 5 versus uninfected PC12 cells. However, there was also a statistically significant cell death at this MOI when compared to uninfected control cells. Similar cell toxicity was observed with PC12 cells infected with GFP virus at an MOI of 5, indicating a vector-induced toxicity. An intermediate MOI of 3 was included and it showed a similar level of GAD67 up-regulation with no significant cell toxicity. The ATF G3

was further validated *in vivo*. Rats were unilaterally lesioned using 6-hydroxydopamine (6-OHDA). Lentiviral vectors containing G3 or GFP were injected bilaterally, at 3 sites in striatum, 3 weeks after the lesion. The rats were sacrificed after 3 additional weeks and the individual striata were dissected and divided into smaller sections. The level of GAD67 protein was determined by Western blot. Up to a 3.5 fold up-regulation of GAD67 protein was observed in striatum of rats injected with G3 virus when compared to rats that were lesioned only. No up-regulation of GAD67 protein was observed following injection of GFP virus. These results suggest that ATFs can be used to up-regulate endogenous GAD67.

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Axonal pathfinding in the developing nigrostriatal projection system

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The ascending dopaminergic projection system originates in the substantia nigra (SN) during the late embryonic stage and matures during the early postnatal period. Although the birth and differentiation of mesencephalic dopaminergic neurons is relatively well characterized, less is known about the molecular and cellular mechanisms, which govern the axon guidance and pathfinding, dendrite formation and maturation of the dopaminergic projections system of the nigrostriatal pathway. Recent studies on axonal pathfinding during development and regeneration within the CNS have unravelled a number of interesting candidate molecules, which guide or prohibit axonal outgrowth. In this context, cell transplantation has been used previously as an important methodological tool to study the inherent plasticity of neuronal systems. For example, it has been shown that the axons of transplanted neurons can be guided *in vivo* by ectopic expression of guidance cues. Ectopic grafting of embryonic ventral mesencephalon (VM) tissue into the lesioned striatum in the adult 6-OHDA rat model of PD can partially restore DAergic neurotransmission in the transplanted area. However, if transplanted homotopically into the adult rat's SN, VM grafts are incapable of reconnecting with the striatum. In contrast, anatomically correct reinnervation of the striatum by intranigral transplantation followed by long-distance axonal outgrowth is feasible in the neonatal rat model of PD. Importantly, the reformation of the nigrostriatal pathway is dependent on the age of the host animal and can occur only during a limited postnatal period. We have investigated the postnatal time-window in which axonal pathfinding from the SN, along the medial forebrain bundle, into the striatal target area was possible. Using the neonatal 6-OHDA rat model, anatomical and functional restoration by intranigral grafting of E14 VM cells could be demonstrated in young rats until postnatal day 10–12 (P10–P12); but not anymore after P20. The aim of the current project is (i) to identify the critical time-window more exactly and (ii) to examine the mechanisms and molecular substrates, which influence the organotypic axonal pathfinding of intranigral dopaminergic neurons. RNA *in situ* hybridisation and immunohistochemical methods will be employed to the extent of axonal reconnectivity of the nigrostriatal pathway. Lesioned and/or transplanted animals will also be tested on behavioural paradigms such as drug-induced rotation, the cylinder test, and the corridor test. Pathway reconstruction analysis will be facilitated using

tissue donors expressing GFP and by applying anterograde and retrograde tracers. Furthermore, the chemo-attractive and chemo-repulsive properties of brain regions associated with the nigrostriatal pathway will be studied using co-cultures of mesencephalic explants with explants from the MFB, the striatum, and the cortex, from equivalent developmental stages as well as across days.

Short-term shift from low to high oxygen tension promotes dopamine release from differentiated ventral mesencephalic precursor cells

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Effective numerical expansion of dopaminergic precursors might overcome the limited availability of transplantable cells in replacement strategies for Parkinson's disease. For this the oxygen tension may be critical. Physiological oxygen tension in the CNS is in the range of 1–5%, while cell culturing normally is performed at atmospheric and hence high non-physiological oxygen tension. Here we investigated the combined effect of fibroblast growth factor-2 (FGF2) and FGF8 and culturing at high (20%) and low (3%) oxygen tension on expansion and dopaminergic differentiation of rat embryonic ventral mesencephalic neuroblasts. Cell proliferation in terms of bromodeoxyuridine incorporation was higher in cultures expanded at low compared to high oxygen tension, just as neuronal cells were more abundant in cultures differentiated for 6 days at low oxygen tension. Application of low oxygen during FGF2-mediated expansion and subsequent differentiation without FGF2 also significantly increased the number of tyrosine hydroxylase-immunoreactive (TH-ir) dopaminergic neurons, and showed a tendency for a higher percentage of TH-ir cells as compared to high oxygen tension. Notably, however, without eliciting a corresponding effect on K⁺-stimulated dopamine release to the culture medium. Switching FGF2-expanded cultures from low to high oxygen tension during the last two days of differentiation did, however, significantly enhance both K⁺-stimulated dopamine release and intracellular dopamine levels as compared to all other treatment groups. This effect was not found when simply shifting the cultures from low to high oxygen tension during the K⁺-stimulation. The high-oxygen induced increase in dopamine release appeared to be related to an increased *in situ* assessed TH enzyme activity, and not changes in TH-positive cell maturation, cell proliferation or survival. Our findings demonstrate that modulation of oxygen tension can influence not only *in vitro* expansion and dopaminergic differentiation of rat embryonic midbrain precursor cells, but also the cellular level of dopamine.

Multi-tract microtransplantation increases the yield of DARPP-32-positive embryonic striatal cells in a rodent model of Huntington's disease

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Embryonic striatal tissue mediated functional recovery in the rodent lesion model of Huntington's disease (HD) correlates with the proportion of dopamine- and adenosine 3',5'-monophosphate-regulated phosphoprotein with a molecular weight of 32 kDa (DARPP-32)-positive neurones in the graft. The current study investigated the impact of the microtransplantation procedure by comparing the DARPP-32-positive cell numbers in the grafts following either single-tract or multi-tract cell delivery protocols. Cells derived from the whole ganglionic eminence of E15 rat embryos, ubiquitously expressing green fluorescent protein (GFP), were implanted into unilaterally QA-lesioned rat striatum either as 2 x 1.8 µl deposits in a single-tract, or as 18 x 0.2 µl deposits disseminated over six needle, multi-tract, penetrations. For both groups, an ultra-thin glass capillary with an outer diameter of 50 µm was used. Histological assessment at 4 months after transplantation showed nearly two-fold increase of DARPP-32-positive striatal like neurones in the multi-tract compared to the single-tract group. Multi-tract grafts tended to have larger overall volumes, increased DARPP-32-positive zones and were more extensively innervated by dopaminergic projections. However, the cellular make-up of the grafts did not translate into functional differences as tested in simple spontaneous behaviour tests. The results show that distribution of fetal striatal tissue in multiple submicroliter deposits provides for an increased yield of striatal-like neurones, potentially due to the enlargement of the graft-host border area intensifying the graft's exposure to host-derived factors. Furthermore, the use of embryonic tissue from GFP donors was validated in cell-based therapy studies in the HD model.

Behavioural and histological analysis of a partial double-lesion model of MSA-P

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Multiple system atrophy (MSA) is a neurodegenerative disease characterised by progressive autonomic failure, cerebellar ataxia (MSA-C) and parkinsonism (MSA-P) due to neuronal loss in multiple brain areas associated with oligodendroglial cytoplasmic α -synuclein inclusion bodies. There are no effective treatments for MSA, and MSA-P patients are not even responsive to L-DOPA due to the loss of striatal dopaminergic post-synaptic receptors. Rendering MSA-P patients sensitive to L-DOPA administration following striatal tissue transplantation is now considered as a clinical option to manage the disease. The study describes the simple, skilled, and sensorimotor behaviour deficits in a unilateral partial double-lesion rat model of MSA-P. The double-lesion combines a partial/terminal 6-OHDA lesion, followed by a striatal quinolinic acid (QA) lesion and it aims to mimic the pathology of early stage of MSA-P. Animals received baseline

training on the staircase and the corridor tests, and were additionally tested on the stepping, the cylinder, and the drug-induced rotation on multiple occasions following lesion surgery. Under the surgical and testing paradigms used, the behavioural data shows robust lateralised deficits on all tasks, albeit the partial 6-OHDA and the double-lesioned animals were most impaired. Interestingly, the double-lesion had an accumulative effect on the corridor test, partially on the staircase test, and altered deficit profile compared to the two other lesion groups on the rotation and the staircase tests. Histological analysis confirmed the approx. 40% dopamine loss from the striatum (6-OHDA and double-lesion animals), as well as the similar loss of striatal neurones (QA and double-lesion animals). In summary, the study generated the behavioural deficit profile of a partial double-lesion rat model mimicking the early stage of MSA-P. Using this platform, we will transplant embryonic ganglionic eminence cells into the double-lesioned striatum to investigate the degree and conditions under which L-DOPA sensitivity can be re-established.

Application of genetically modified neuronal progenitor cells in a rat model of Parkinson's disease – validation of the co-layer method using a BDNF-expression plasmid

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Primary symptoms of Parkinson's disease (PD) result from loss of dopaminergic (DA) neurons in the substantia nigra. Exogenous cell replacement represents a potent treatment option for this neurodegenerative disorder, despite several limitations, such as lack of donor tissue and low survival of grafted cells. To improve the transplantation outcome, we established a method to combine transient genetic modification of neuronal progenitor cells (NPCs) with an optimized cell culture protocol prior to intrastriatal transplantation into 6-OHDA unilateral lesioned rats. NPCs obtained from the ventral mesencephalon of E12 rat embryos were in vitro proliferated, nucleofected and differentiated as previously described (Timmer et al. Neurobiol. Dis. 2006; Cesnulevicius et al., Stem cells 2006). Brain-derived neurotrophic factor (BDNF) and enhanced green fluorescence protein (EGFP) were expressed with a C-terminal 3xFLAG epitope tag to allow a sensitive detection. Western blot analysis confirmed the functionality of the BDNF-FLAG protein by phosphorylation of BDNF-receptor TrkB after NPCs were incubated with BDNF-FLAG conditioned media. Further, plasmid-based delivered BDNF-FLAG increases the number of tyrosine hydroxylase positive (TH+) neurons by 25% in vitro compared to EGFP transfected controls. However, the nucleofection procedure itself, especially the cell detachment, decreases the number of TH+ neurons to 40% compared to non-transfected sister cultures. To circumvent this drawback we established the co-layer method, which contains a mix of detached and nucleofected cells reseeded on top of an adherent sister culture in a ratio 1:3. In this setup TH+ neuron number remained high and was 25% increased after BDNF-FLAG transfection in vitro. Comparison of both cell culture procedures (standard and co-layer) after intrastriatal transplantation revealed similar DA neuron survival as in vitro. Two weeks after grafting TH+ neuron number was strongly reduced in the standard group (271 ± 62) compared to 1723 ± 199 TH+ neurons in the co-layer group. In contrast to the in vitro results, no differences in the number of grafted TH+ neurons were observed between BDNF-FLAG, EGFP-FLAG and non-transfected co-

layers, neither 2 nor 12 weeks after transplantation. Likewise, amphetamine induced rotation behaviour improved similarly over time in all groups. Interestingly, even 13 weeks after transplantation EGFP-FLAG expression was still detectable in few neurons and an abundant neurite branching inside the grafts could be visualized by anti-FLAG staining, whereas axons of TH+ neurons innervated also the host striatum. Nevertheless, the co-layer protocol provides an efficient way for neurotrophic factor release by transplanted progenitor cells and will be used to study the effects of promising factors on survival and integration of transplanted dopaminergic neurons.

PET imaging and microdialysis studies help explain how cell transplants may interfere with dyskinesias

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Parkinson's disease (PD) is characterized by loss of dopamine in the striatum secondary to the degeneration of the nigro-striatal projection neurons. Proof-of-principle studies in animal models have shown that fetal tissue dissected from the ventral mesencephalic region at a particular time of embryonic development contained neuroblasts that could survive the transplantation process and give rise to dopaminergic neurons after a period of maturation in the host brain. Although early open-labelled trials in small numbers of patients suggested that the outcome of primary ventral mesencephalic tissue grafting could be positive at least in some cases, placebo-controlled double blind studies were essentially negative and showed that several patients had worsening dyskinesias after receiving the grafts. This was an unexpected finding; one that generated a need to go back to animal studies and test new hypotheses. In fact, recently generated data in animal studies, where graft-induced dyskinesias were modelled, we found that the abundance of serotonin neurons in the grafted cell mix could be one of the important determinants of how dyskinesias may be altered after grafting. In follow-up work we have shown that the serotonin cells stored and released dopamine after peripheral L-DOPA treatment but failed to correct the deficit in synaptic DA pool, as evidenced by 18F-fallypride PET studies suggesting that the site of DA release from serotonin cells were inappropriate.

Immune response after cerebral engraftment of human neuronal cells in patients with Huntington's disease

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Diseases of the central nervous system, particularly those of genetic, metabolic, or inflammatory etiology, are associated with neural degeneration or dysfunction. Neuronal cell transplantation is under consideration to treat some of these disorders. Furthermore, who are treated for brain tumors experience significant long-term deficiencies in motor functions. In some, the therapy might also require neural cell replacement, a challenge not regarded as amenable to cell replacement treatment. The allogeneic graft material in human brain raises questions of immune response. Very little data is available how the human

brain reacts immunologically to cell transplantation over time. Anti HLA-antibodies may act as an early indicator of a slowly emerging rejection that is not yet manifested clinically. We measured in patients with Huntington's disease immune responses as assessed by the presence of anti HLA-antibodies to allogeneic intra-striatal transplantation of fetal neurons. The patients received standard triple immunosuppressive therapy for one year after stereotactic intervention. Out of 10 patients five developed anti HLA-antibodies of class I and II. The time between transplantation and development of HLA antibodies was variable, with detection either immediately after engraftment, during immunosuppressive therapy, or three and 24 months after prophylactic immunosuppressive therapy was stopped. The antibodies disappeared in three patients when immunosuppression was reinstated. Development of anti HLA-antibodies had no immediate effect on the initial favourable clinical response. The findings show that there is indeed a significant number of patients who may develop antibodies to allogeneic cell transplantation into the brain parenchyma. The triggering of anti HLA-antibodies may not lead to immediate graft rejection, a possibility that cannot be excluded. As the time of onset of immune response is unpredictable, long term prophylactic immune suppression cannot be justified. Instead regular assessment of blood for anti HLA-antibodies for indefinite long periods appears mandatory. Given the ongoing cell transplantation studies for Huntington's and Parkinson's disease, and future cell transplantation programs with brain tumors, the results are of significant implications.

Hibernation of ventral mesencephalon affects graft innervation and post-transplantation dyskinesia

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The effective transplantation of embryonic dopaminergic ventral mesencephalon requires a critical number of dopaminergic neurons. The logistics of obtaining the necessary 4–6 human embryos per transplanted hemisphere requires that some tissue may have to be stored for a few days prior to transplantation, a process which has been used where necessary in some clinical trials. Although studies on tissue storage have been carried out in the past, the problems of post-transplantation dyskinesia had not been highlighted. We therefore examined the effect of 8 days hibernation vs fresh tissue on functional outcome, L-dopa and amphetamine-induced dyskinesia and graft survival. Three groups of 6-hydroxydopamine lesioned Sprague Dawley rats were treated with L-DOPA (10 mg/kg plus benserazide 15 mg/kg s.c.) daily for 3 weeks. Embryonic day 14 ventral mesencephalon was dissected and stored as pieces in hibernation media at 4 °C for either 24 h 'fresh' or 8 days 'stored'. Rats were then transplanted with 200 000 cells from a suspension made from one of the tissue samples or injected with saline as control. Motor and AIMs assessments were carried out pre-transplantation, and at 6, 12 and 18 weeks post-transplantation. Histological assessment of the graft showed a non-significant trend for lower tyrosine hydroxylase positive cells within the stored grafts compared to fresh, with a smaller area of graft innervation. The average TH density over the striatum was equal between grafted groups but the stored grafts innervated more medial areas of the striatum whilst fresh grafts innervated more lateral regions. Few 5-HT positive cells were identified in either graft type. Amphetamine-induced rotations were reduced to a similar degree in both transplant groups and both showed a reduction in L-DOPA-induced AIMs.

However, only the fresh transplant group showed significant amphetamine-induced AIMs. The most interesting feature of this study was the differential distribution of innervation from stored and fresh grafts, to more medial or lateral regions of the striatum respectively. This, in addition to the larger and more widely innervating histology of the fresh grafts may explain the higher level of amphetamine-induced AIMs observed in this group. Importantly this study indicates that if cell loss is adjusted for, although striatal reinnervation may be more limited, storage of graft tissue does not appear to negatively impact on behavioural outcome or dyskinesia presentation.

Optimising transplantation of novel iPSC cells in a rodent model of Huntington's disease

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Ethical and logistical issues concerning the use of primary fetal tissue and ES-derived stem cells for transplantation in Parkinson's and Huntington's disease have enforced the need to develop novel cell sources that are safe, consistent and renewable. The recent development of iPSC cells provides a potential resolution. However, the safety, viability and phenotypic characteristics of different iPSC cell lines need to be investigated thoroughly after *in vivo* transplantation. Furthermore, the influence of several transplantation parameters have yet to be investigated. To this end, one line of GABAergic iPSC cell (AF22) was used to compare the impact of transplanting FACS sorted versus unsorted cells and the effect of grafting into a lesioned versus an intact host system. Results suggest that the AF22 cell line survives well *in vivo* and no safety issues were evident. The grafts formed from unsorted cells were found to show better survival and greater volume than those observed for FACS sorted cells. Furthermore, transplantation into the lesioned hemisphere was found to be conducive to good graft survival and integration, while transplantation into the intact brain resulted in fewer surviving cells and smaller grafts. Current experiments aim to compare the AF22 cell line to a second GABAergic iPSC cell line. Overall, although many parameters of transplantation have yet to be systematically optimised, the results of these experiments suggest that iPSC cells may provide a potential source of cells for use in cell replacement therapies for Huntington's disease.

The efficacy of intrastriatal carotid body (CB) grafts in a new chronic MPTP mouse model of Parkinson's disease correlates with CB GDNF expression, which is differentially regulated by chronic hypoxia along aging

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Previous work from our laboratory shows that intrastriatal transplantation of dopaminergic carotid body (CB) cells produce an amelioration of parkinsonism in animal models mainly due to a trophic effect, and can also induce significant beneficial clinical effects in Parkinson's disease (PD) patients. The mechanisms that underlie the trophic effects mediated by CB cells over the nigral neurons remain unknown. In order to study these mechanisms we have developed a new systemic chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model that better resembles the features of PD, without the

high mortality and variability inherent to other MPTP protocols. In this model of parkinsonism we have studied: i) stereological and densitometric analysis of the nigrostriatal pathway after TH- and NeuN-immunohistochemistry, ii) striatal dopamine content determinations by HPLC, and iii) behavioral tests. Unilateral striatal CB transplants with a sham graft in the contralateral striatum were performed in this animal model. Besides these grafts we used fluorescent latex beads to trace in a retrograde manner the neurons of the SN that were in contact with the transplanted CB cells. Intrastriatal CB grafts clearly protected MPTP-induced degeneration of SN dopaminergic neurons. Confocal microscopy analysis showed that the protected SN neurons were those exposed to the grafted CB cells. The trophic effect that intrastriatal CB grafts induce on nigrostriatal neurons can be explained because CB glomus cells produce large amounts of Glial cell line neurotrophic factor (GDNF). Moreover, CB cells are physiologically resistant to hypoxia, a normal environmental condition in the brain that is accentuated inside intracerebral grafts. Here, we report that chronic hypoxia induce an upregulation of GDNF expression on CB cells (2–3 months old). Surprisingly, on aged CB (> 14 months) GDNF expression is diminished by chronic hypoxia. This differential regulation of GDNF expression by aging is also observed on intrastriatal grafts. We tested how differential GDNF expression between young and old intrastriatal CB graft affects the efficacy of antiparkinsonian CB cell therapy. Intrastriatal graft of young CB induces an important recovery of MPTP treated mice while old CB graft fail to produce a significant recovery. These findings are in concordance with previous clinical trials, where the efficacy of CB autotransplantation in PD patients was inversely related to patient age. Thus, dopaminergic CB glomus cells appear as an excellent biological pump to produce endogenous delivery of GDNF, but age of CB cells is an important issue that must be taken into consideration for its use in cell therapy.

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Generation of an age- and region-specific map of the developing central nervous system to characterize cells for neurotransplantation

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Detailed analysis of the composition of neural precursors for cell transplantation is a critical process since contamination of cells from other brain regions is undesirable and might mitigate the functional benefits. Here we report the establishment of a marker-based map that allows the analysis of the composition and developmental age of cultured neural precursors by using immunocytochemistry. E14 Sprague-Dawley rat embryos were dissected into cortex (dorsal telencephalon), ganglionic eminence (ventral telencephalon), diencephalon, ventral mesencephalon and dorsal mesencephalon (tectum). Cells were enzymatically dissociated and cultured under differentiation conditions for 24 h and 7 days. Afterwards wells were processed for immunocytochemistry, using foetal brain region-specific transcription factors to analyse neural precursors and neurotransmitter-phenotype associated markers to analyse mature neurons. In addition, well characterized glial and general neuronal markers as well as markers of the VM and WGE were used. We identified markers for the foetal and adult cortex, WGE, thalamus, and

VM that did not cross stain with other brain regions, or their expression patterns clearly differed from the profile in other regions. Additionally, tissue inclusions from adjacent brain regions of the target region can now be identified with certainty. Extension of this marker tool for the application on human foetal neural precursors is currently in progress, but the data are comparable with the results obtained from foetal rat tissue. In summary, we established a set of markers to analyze differentiation stage and region specificity of cultured neural precursors which will provide an essential tool in future applications of cell-based therapies in clinical neurotransplantation studies.

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Olfactory deficits in an overexpressing α -synuclein mouse model of Parkinson's disease

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Introduction: Parkinson's disease (PD) is a neurodegenerative disorder, which is characterized by the presence of Lewy bodies consisting mainly of α -synuclein aggregates. In addition to motor dysfunctions, PD patients exhibit non-motor symptoms, such as alterations in olfaction, memory deficits and depression. **Aim:** We wanted to study the effect of the accumulation of α -synuclein on olfaction in Parkinson's disease. **Methods:** For this purpose, we used a novel transgenic mouse model, with overexpression of human wild-type α -synuclein under the control of the partial mouse α -synuclein promoter, which has a high expression in many cortical and olfactory areas. We performed an olfactory study employing different tests to evaluate the olfactory function. We studied their ability to detect odors, their short-term olfactory memory, as well as their ability to discriminate odors that could be social or non-social. **Results:** We showed that the odor detection threshold of transgenic mice appears to be higher compared to control mice. At a concentration where they can detect a smell, we observed that the short-term olfactory memory of the transgenic mice is less efficient compared to the control mice. Finally, using time intervals at which mutant mice are not impaired, we demonstrated that transgenic mice are impaired in their ability to discriminate between social odors as well as between non-social odors. **Conclusion:** In conclusion we showed that in a novel mouse model of Parkinson's disease, the over-expression of the human wild-type α -synuclein leads to alterations of different aspects of the olfactory function.

Microbiological safety of fetal tissue in neurorestorative therapies

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Neurorestorative therapies currently apply fetal derived tissue obtained from routine elective abortions. During this procedure, fetal tissue comes into contact with surfaces which naturally carry a biofilm, like the epithelium of the female genital tract, and the potential exposure to microbes from different sources can result in a contami-

nation of the transplanted tissue. We analysed the microbiological contamination in transportation and washing fluids at different time points of the procedure by cultivation on Columbia blood agar. Specimen for incubation were taken from the original transportation media (sample A) and from washing fluids of a first washing after dissection of tissue (sample B) and a third sample after washing following up to 24 hours of hibernation (sample C). Moreover, we analysed the capacity of our washing system to reduce or eliminate contaminations by addition of microbes to sterile rat embryonic structures. This standard testing battery for tissue therapies included *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium sporogenes*, *Corynebacterium amycolatum* and *Candida albicans* as well as *Lactobacillus Jensenii*. The spectrum of microbes found in the human fetal transportation fluid comprised mostly of *Corynebacterium* spp. and *Staphylococcus* spp. Contaminations were detected in a substantial number of transportation media, but after subsequent washing, in B- and C-samples, no microbes were detected by incubation. In the samples taken from artificially contaminated rat tissues, washing resulted in reduction of the number of microbes, but only with *Clostridium sporogenes* and *Candida albicans* did the washing lead to complete removal of measurable contamination. Our data indicate the need of washing steps for the reduction of microbiological contamination in fetal tissues for cell therapies in the brain. In human tissue samples, no microbes were found after washing, but validation of washing efficiency on rodent tissues shows the limitations of the washing procedure. Therefore, the use of microbiologically active substances should be discussed.

Characterisation of FoxP1 as a marker of mature medium spiny neurons and their precursors

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The neuropathological characteristics of Huntington's disease (HD) reveal a predominant loss of medium spiny neurons (MSN) of the striatum. 90% of all mature striatal MSNs express DARPP-32, and although DARPP-32 expression is not absolute in defining a MSN, it is presently the only marker which distinguishes these neurons from other striatal cells. A microarray study, carried out in our lab looking at gene expression in the developing striatum showed that FoxP1 expression in the putative striatum, or whole ganglionic eminence (WGE), was up-regulated between the gestational ages E12 and E16. These findings were confirmed by *in situ* hybridization and QPCR analysis, and prompted investigation of FoxP1 as a potential marker for striatal cells including MSNs (Vinh et al., unpublished). We have subsequently looked at further characterising FoxP1 as a marker of MSNs and their precursors, using *in vitro* cell culture of embryonic WGE from mouse, rat and human, and histological examination of the rodent adult brain both intact and post-lesion and transplantation. *In vitro* analysis of differentiated WGE cells dissected from E14 brains revealed co-expression of FoxP1 with markers of neurons but not astrocytes. Furthermore, we show co-expression of FoxP1 with the striatal projection neuron markers DARPP-32 and met-enkephalin, and no co-expression with the interneuron marker parvalbumin. We also carried out immuno-fluorescence on cultures of WGE taken from human fetal brain, retrieved from routine elected termination of pregnancy clinics. We demonstrated expression of FoxP1 in short-term differentiated neurons from human fetal tissue, but no expression of DARPP-32 within these 'early' differentiating cultures. A developmental screen revealed that FoxP1 expression remains elevated through to

adulthood. In the adult brain FoxP1 expression is not restricted to the striatum, and analysis of the whole adult brain revealed its expression not only throughout the striatum, but also in the cortex and hippocampus. However, within the striatum, expression is restricted to neurons, with co-expression of FoxP1 with projection neurons but not interneurons. In addition to FoxP1 as a marker of MSNs in normal development, we have gone on to look at its use as a marker of phenotypes within developing grafts using mouse and rat tissue as both donor and host. Administration of the excitotoxin quinolinic acid (QA) directly to the striatum revealed that as well as loss of DARPP-32 immunopositive neurons, there is a loss of FoxP1 expression throughout the depleted striatum. Histological analysis of allografts of E14 WGE cells into the QA-lesioned striatum revealed cells expressing FoxP1 at different time points post-transplantation. These findings are encouraging for the use of FoxP1 as a striatal marker in addition to DARPP-32. In particular, FoxP1 may identify developing MSNs at a much earlier stage, which will be important when generating MSNs from novel cell sources.

Immobilization mask to reduce movement artefacts in MRI images

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Objective: In order to improve image quality in a synchronized fMRI-EEG study with patients suffering from the involuntary movements typical for Huntington's disease, the requirement was to develop a technique for immobilizing the heads of our patients inside an MRI head coil in order to assure a good image quality. **Methods:** To develop the mask, we modified a technique used to fabricate masks used for reliable repositioning in temporally fractionated radiotherapy. Finally, we tested the mask in five patients with Huntington's disease, acquiring both fMRI and EEG signals in a synchronized setting as well as structural (T1) MR images. **Results:** Image quality as well as EEG-signal quality was significantly improved in all patients wearing the mask. **Conclusion:** We have succeeded in developing a mask that fits into the MRI head coil, does not disturb the MRI signal and significantly improves both MRI image and EEG signal quality.

The role of noradrenergic neurons in motor symptoms of Parkinson's disease and development of L DOPA-induced dyskinesia

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Parkinson's disease (PD) is a neurodegenerative disease associated with a profound loss of dopaminergic (DA) neurons in the substantia nigra (SN); therefore, current therapeutic strategies are focused on restoration of DA neurotransmission or DA neuron replacement. However, studies of postmortem PD brains have shown that not only DA neurons in the SN but also noradrenergic (NA) neurons in the locus ceruleus (LC) degenerate, and that the NA neurodegeneration may be as profound, and also precedes degeneration of the midbrain DA neurons. The early involvement of the NA system is also in line with the caudal-to-rostral disease progression predicted by the model

proposed by Braak et al. Clinically, the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) has been the most common and successful drug to treat the symptoms of PD. However, prolonged administration of L-DOPA produces uncontrolled excessive movement called dyskinesia. We have been investigating the role of NA neurons in motor deficit and development of L-DOPA-induced dyskinesia (LID). Rats were unilaterally lesioned with 6-OHDA with/without desipramine (NA transporter blocker, which protects NA neurons from 6-OHDA toxicity). Two more groups of rats also received DSP-4 injection (a specific toxin which damages NA neurons in LC) before or after the 6-OHDA lesion. Animals have been tested in a battery of behavioral tests such as amphetamine-induced rotation, stepping test, and cylinder test. Rats were given daily L-DOPA injection and abnormal involuntary movements were scored. In amphetamine-induced rotation and cylinder tests, there was no significant difference in the degree of motor deficit between groups. However, the double-lesioned (DSP-4 injected) group showed a significant deficit using affected paws in stepping test than the NA-protected (desipramine injected) group. At 6 mg/kg of L-DOPA injection, only a few animals showed dyskinesia. When 24 mg/kg or 50 mg/kg of L-DOPA was injected, rats that received desipramine to protect the NA system showed significantly less dyskinesia. The analysis of the level of DA and NA in cortex and striatum is on-going using high performance liquid chromatography. So far, only a few studies have reported the influence of NA denervation on the development of L-DOPA-induced dyskinesia, with contradicting results. In fact, one study found increased susceptibility, while another one showed no difference between single (dopamine only) and double (dopamine plus NA) lesioned rats. Our data indicates that impairment of NA system worsens the degree of motor deficits and makes rats more prone to develop L-DOPA-induced dyskinesia.

Are astrocytes required for optimum survival of embryonic stem cell-derived dopaminergic transplants?

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Despite much progress over recent years in the development of protocols for the induction of dopaminergic neurons from ES cells, the reports that are available on transplantation of such ES cell-derived neurons into Parkinson's models show that survival is less robust and more variable than with primary dopaminergic neurons and does not achieve full recovery of rotational asymmetry, the most easily saturated test of graft function. We hypothesize that the protocols used force the differentiation of early progenitors into neurons at the expense of astrocytes. Mesencephalic astrocytes specifically are known to support the survival and differentiation of dopaminergic neurons, and their absence is likely to negatively affect viability/function of dopaminergic grafts. Our preliminary investigation into this subject indicates that at early time points following transplantation, primary human fetal ventral mesencephalic grafts contain donor-derived differentiated astrocytes, whereas ES cell-derived grafts do not. Such astrocytes may support dopaminergic neuronal survival not only through the secretion growth factors and extracellular matrix proteins, but also through other means such as gap junction coupling. Once the differences between astrocytes in primary and ES cell-derived dopaminergic grafts are closer defined, astrocytes from alternative cell sources may offer an efficient way of improving the effectiveness of ES cell-derived grafts.