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Assessment of the PhD dissertation by
Marián Hruska-Plochán, M.Sc.

”Huntington’s disease modeling and stem cell therapy in spinal cord disorders and injury”

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By:

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Supervision, place of study and international collaboration
The thesis originates from the Institute of Animal Physiology and Genetics AS CR, v.v.i., Libechov. Professor Jan Motlik, DVM, D.Sc has been the main advisor. Marian Hruska-Plochan has performed a part of the study at University of California San Diego, CA, USA, under the supervision of Consultant Professor Martin Marsala, MD.

Composition of the Ph.D. Thesis
The thesis based on five papers and consist of 274 pages, whereof 47 pages are introduction & aim, 7 pages material and methods, 160 pages results (the five papers), 16 pages discussion & conclusion, 30 pages references, and 10 pages abstract, abbreviations, author publications & CV. There are 17 figures and 2 tables in the main text (excluding the papers) of the thesis.

The 5 papers are:

I) Baxa M, **Hruska-Plochan M**, Juhas S, Vodicka V, Pavlok A, Juhasova J, Miyanohara A, Nejime T, Klima J, Macakova M, Marsala S, Weiss A, Kubickova S, Musilova P, Vrtel R, Sontag EM, Thompson EM, Schier J, Hansikova H, Howland DS, Cattaneo E, DiFiglia M, Marsala M, Motlik J (2013) A transgene minipig model of Huntington’s disease. **Journal of Huntington’s Disease** 2:47-68.

II) **Hruska-Plochan M**, Juhas S, Juhasova J, Wu S, Dumpi J, Weiss A, Marsala M, Motlik J (submitted) Partial UCHL1 depletion in R6/2 mouse model of Huntington's disease accelerates mutant huntingtin aggregation. **Neuroreport** (submitted)

III) Hefferan MP, Galik J, Kakinohana O, Sekerkova G, Santucci C, Marsala S, Navarro R, **Hruska-Plochan M**, Johe K, Feldman E, Cleveland DW, Marsala M (2012) Human neural stem cell replacement therapy for amyotrophic lateral sclerosis by spinal transplantation. PLoS One, 7(8):e42614

IV) van Gorp S, Leerink M, Kakinohana O, Platoshyn O, Santucci C, Galik J, Joosten Ea, **Hruska-Plochan M**, Goldberg D, Marsala S, Johe K, Marsala M (2013) Amelioration of motor/sensory dysfunction and spasticity in a rat model of acute lumbar spinal cord injury by human neural stem cell transplantation. **Stem Cell Research & Therapy** (accepted)

V) Seve J, Goldberg D, van Gorp S, Leerink M, Juhas S, Juhasova J, Marsala S, **Hruska-Plochan M**, Hefferan MP, Motlik J, Rypacek F, Machova L, Kakinohana O, Santucci C, Johe K, Lukacova N, Yamada K, Bui JD, Marsala M (in revision) Effective long-term immunosuppression in rats by subcutaneously implanted sustained-release tacrolimus pellet: effect on spinally grafted human neural precursors survival. **Experimental Neurology** (in revision)

Written statements from professor Jan Motlik declare that Marian Hruska-Plochan was adequately involved (70%) in the study, design, experiments and interpretation of results and writing of paper I-II.
Professor Martin Marsala declare in a similar way that Marian Hruska-Plochan was adequately involved (30%) in the study, design, experiments and interpretation of results and writing of paper III-V.

Background for the thesis and the results
This thesis project aims to:

1A (paper I): Generate and characterize a transgenic minipig of Huntingtons's disease (HD)

1B (paper II): Study the potential role of UCHL1 in ubiquitin proteasome system impairment in HD

2A (paper III): Test the potential therapeutic effect of spinal grafted human neural stem cells on the disease progression in the $SOD1^{G93A}$ ALS rat model

2B (paper IV): Examine amelioration of motor and/or sensory dysfunction and spasticity in a rat model of acute lumbar spinal cord injury by human neural stem cell transplantation

2C (paper V): Develop a more reliable and less labour intensive immunosuppression protocol for xenogenic neural stem cell transplantation experiments in rats

The thesis will accordingly try to improve the understanding of Huntington's disease, and the stem cell based treatment options in Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and traumatic spinal cord injury, which are grave frequent disorders of the central

nervous system with dramatic socioeconomic consequences. Good and curable therapies are, however, lacking for these deadly (HD and ALS) or frequently severely disabling disorders. Thus, there is a permanent or progressive loss of nerve cells in the brain or spinal cord causing the disease symptoms, while existing medication cannot give sufficient symptomatic relief. One emerging therapy based on replacement of the lost neurons and/or restoring of disrupted connectivity by intra-spinal cell transplantation, is accordingly investigated in the current thesis. Such treatment may also result in the release of trophic factors or buffering of disease causing chemical imbalances in the CNS parenchyma by the transplanted cells.

In the first part (chapters 1,3-6, papers I-II, aims 1A-B) of the present thesis work, the aim is to generate and characterize a new large animal model of HD for the future development of new disease modifying therapies by lentiviral insertion of a construct encoding for the first 548 amino acids of the human huntingtin (HTT) protein under control of human HTT-promoter into porcine zygotes resulting in a transgene minipig model of HD. The resulting F0-F3 generations were subsequently characterized by motor scores, biochemical assays, fertility and semen examinations, and post-mortem immunohistochemistry and HTT-aggregate analysis. This question of mutant huntingtin aggregation and disturbed protein homeostasis in HD is then explored in an experiment where a mouse model of HD (R6/2) is crossed with a mouse model (gad) which lack expression of the deubiquitinating enzyme UCHL1.

In the second part (chapters 2-6, papers III-V, aims 2A-C) of the present thesis work, the aim is to examine the therapeutic potential of xenogenic neural stem cell transplantation in spinal cord disorders and injury. This is done by intra-spinal grafting of human neural stem cells in a $SOD1^{G93A}$ mouse model of ALS and in a rat model of compression induced acute lumbar spinal cord injury. A method of tacrolimus based immunosuppresion for xenograft studies in rats is finally developed and examined.

The individual chapters & papers

Chapter 1

In the introduction to this part of the thesis dealing with Huntington's disease Marian Hruska-Plochan clearly delineates the field including the clinical and genetical background, putative pathogenesis covering excitotoxicity, mitochondrial dysfunction, translational dysregulation, proteolysis, UPS-dysfunction, autophagy, protein misfolding and aggregation, together with the current therapeutical approaches and the current transgenic animal models of HD. One could argue that some parts are a little too short, such as the role of UCHL1 in the UPS-cascade. There are also some misinterpretations in the historical presentation of HD disease, where initial cases of the dancing mania probably were caused by a fungus contamination of grain as hole villages developed symptoms at the same time. Likewise more concern could be stated for the effect of localized treatments (grafting, viral vector injection etc) against a generalized CNS disorder. Increased emphasis on the Chinese transgene minipig model of HD (Yang et al., 2010) would likewise be pertinent as their model apparently is symptomatic very rapidly in contrast to the actual presented Czech minipig model. However, overall the HD-introduction is well organized, gives appropriate references and focuses on the issues relevant for this thesis work including a detailed description of putative causes of HD and current transgene models.

Chapter 2

In the introduction to this part of the thesis dealing with neural stem cell therapy in spinal cord disorders and injury Marian Hruska-Plochan introduces neural stem cell therapy in the spinal cord. This is followed by a thorough description of ALS delineating the field including the clinical and genetic background, neuropathology, existing animal models with special

emphasis on the SOD1 models but also including new genetic findings and putative pathological mechanisms covering oxidative stress, mitochondrial dysfunction, apoptosis, glutamate excitoxicity, protein aggregation, neuroinflammation, impared axonal transport, glial cell pathology, dysregulated transcription and RNA processing, followed by current therapeutical approaches and the potential of neural stem therapy in ALS. This is followed by a section on spinal cord injury, its current therapy and the potential of neural stem cell therapy in spinal cord injury. Finally, a section delineates the field of immunosuppression in spinal cord stem cell transplantation experiments. This part would likewise benefit from more concern stated for the ability of neural transplantation not only to restore cell number but also to restore peripheral and central connectivity which is the main problem in spinal cord restoration. However, care is taken to point out that local cell transplantation may fill out tissue defects, lead to functional by-pass connectivity, and to the release of trophic factor of benefice for the spinal cord environment. Thus, this chapter is well organized, gives appropriate references and focuses on the issues relevant for the subsequent parts of the thesis.

Chapter 3

State the thesis aims which are referred to above.

Chapter 4

This chapter gives an overview of the material and methods used in the thesis, whereas more detailed information of each procedure is given in the subsequent papers upon which the thesis is based. Chapter 4 is much needed as the number of animal species and methods used are impressive high. Thus, three animals species are used e.g. minipig, mouse, and rat. A transgene minipig model is developed, a mouse ALS model is crossed with a mouse UCHL1 deficient mouse, and studies are likewise performed on a rat ALS model and a rat model of spinal cord injury. The used methods span from vector construction and transgenesis, to stem cell handling, grafting, immunosupression, electrophysiology, high field MRI, biochemical assays, and immunofluorescence and immunohistochemical stainings. This chapter is concluded with an overview of the statistical methods used.

Chapter 5 (paper I)

The first paper (in chapter V) with the title “A transgene minipig model of Huntington’s disease” is published internationally in the Journal of Huntington’s Disease with Marian Hruska-Plochan as joined first author.

The study is based on lentiviral insertion of a construct encoding for the first 548 amino acids of the human huntingtin (HTT) protein under control of the human HTT-promoter into porcine zygotes resulting in F0-F3 transgene minipig generations, that were subsequently characterized by motor scores, biochemical assays, fertility and semen examinations, and post-mortem immunohistochemistry and HTT-aggregate analysis. The authors show that one copy of the human HTT transgene encoding 124 glutamines were integrated into chromosome 1 q24-q25 with successful transfer through all successive generations. Mutant HTT mRNA and protein fragments were detected in brain and peripheral tissues, however, no protein aggregations were noted and no neurological dysfunction were seen up to 40 months of age. However, a pair of 16 month siblings displayed reduced neostriatal DARPP32 immunoreactivity, and transgene boars had by 1 year of age reduced fertility and fewer spermatozoa pr ejaculate, which likewise displayed reduced ability to penetrate WT minipig oocytes. These findings lead the authors to conclude that a transgene minipig model of HD has been established, useful for long-term safety testing of HD therapeutics, although the emergence of HD-like phenotypes will require more study.

The paper is based on transgenetic work of the highest international standard and subsequent observational, biochemical and postmortem tissue analysis suited to elucidate the

considered scientific problem. The authors have accordingly in my view chosen the right scientific experimental approach to reach the wanted scientific goal. However, the lack of neurological symptoms and convincing CNS pathology is distressing, and I do strongly agree with the authors that further elucidation of the phenotype is required. It would likewise be interesting to compare the current transgene minipig model with a Chinese transgene HD minipig model described in the introduction where the animals appearantly display symptoms extremely fast. Why this difference in phenotype?

Chapter 5 (paper II)

The second paper (in chapter V) with the title "Partial UCHL1 depletion in R6/2 mouse model of Huntington's disease accelerates mutant huntingtin aggregation" is presented as a submitted manuscript to the international journal Neuroreport with Marian Hruska-Plochan as first author.

The study examines by crossing of the R6/2 HD mouse model with the gad mouse model which lack expression of the deubiquitinating enzyme UCHL1, the role of the Ubiquitin Proteasome System (UPS) in HD. The resulting animals where weighed and the removed brains analysed with counting of DARPP32 positive medium spiny neurons in the striatum followed by Time-resolved Förster Resonance Energy Transfer (TR-FRET) qualitative analysis of soluble mutant, wild-type and aggregated mutant HTT, as well as immunofluorescence quantification of mutant huntingtin aggregates. The authors demonstrate quantitatively that the reduction of UCHL1 in the R6/2 mouse significantly accelerated mutant HTT aggregation and increased the levels of polyubiquitin chains and proteins, although it NOT potentiated brain neuronal degeneration. The authors conclude that their results suggest that the UPS in HD might be affected by inefficient recycling of monomeric ubiquitin by the deubiquitinating enzymes, particulary by UCHL1, and that the formation of inclusion bodies is a result of a neuronal disease-coping response to more toxic mutant HTT species.

The paper is based on rigorous scientific testing and methods at the highest international level suited to elucidate the considered scientific problem. Although the working hypothesis e.g. the proposed link between HD and the UPS system seems a bit enigmatic, and not is strengthen by the lack of HD symptoms in the gad mouse model, and the current lacking differences between the R6/2 and the R6/2xgad mouse model. The authors have, however, chosen the right scientific experimental approach to elucidate the proposed relation. The statistical methods chosen are appropriate. The results are presented clearly and discussed in the context of the present scientific knowledge in the field. I am however not fully convinced that the data support the UPS dysfunction and protective role of inclusion bodies hypotheses as stated in the conclusion as no clinical or quantitative neuronal change was noted between the two experimental mouse groups.

Chapter 5 (paper III)

The third paper (in chapter V) with the title "Human neural stem cell replacement therapy for amyotrophic lateral sclerosis by spinal transplantation" is in publication at the international renowned journal PLoS One with with Marian Hruska-Plochan as 8th author.

The study examines by clinical observation, electrophysiology and immunohistochemistry quantitative methods the influence of lumbar spinal cord grafted human fetal spinal neural stem cells (hNSCs) in a rat model of ALS ($SOD1^{G93A}$-rats). The authors show convincingly with beautiful images and electrophysiologic data that grafted hNSCs display long term survival and preferential neuronal differentiation, and some preservation of α-motorneuron survival at lumbar but not at cervical levels, and that the grafted cells make synaptic contacts with host neurons, probably accounting for a transient protection of hind limb motor function and the electrophysiological measured Hoffman reflex. The authors validate furthermore the

$SOD1^{G93A}$-rat model by demonstration of significant end-stage disease degeneration of descending medium and large-size myelinated axons by electrophysiology (motor evoked potentials and histological silver staining. Based on these results the authors conclude that cell transplantation therapy in ALS may be of some benefice but will require multiple grafts targeting both spinal and supraspinal targets.

This paper based on rigorous scientific testing and methods at the highest international level adds valuable new information to the field of neural transplantation in ALS, and the authors should be greatly acknowledged for their thorough and comprehensive work.

Chapter 5 (paper IV)

The fourth paper (in chapter V) with the title "Amelioration of motor/sensory dysfunction and spasticity in a rat model of acute lumbar spinal cord injury by human neural stem cell transplantation" is accepted for publication at the international renowned journal Stem Cell Research & Therapy with Marian Hruska-Plochan as 8th author.

The study examines by clinical observation, electrophysiology, high-field post-mortem MRI and immunohistochemistry quantitative methods the influence of lumbar spinal cord grafted human fetal spinal cord derived neural stem cells (HSSC) in a rat model of L3 spinal cord compression injury. The authors show convincingly with beautiful images and electrophysiologic data that intraspinal grafting led to progressive and significant improvement in lower extremity paw placement, amelioration of spasticity, and normalization in thermal and tactile pain/escape thresholds at 8 weeks post-grafting, probably caused by the development of putative GABA-ergic synapses between grafted and host neurons. MRI volume reconstruction and immunofluorescence analysis of grafted cell survival demonstrate near complete filling of the lesion caused spinal cavity formation. However no significant differences were detected in several other clinical parameters of gait or motor evoked potentials. The introduction is well written, and the materials and methods give a clear presentation of the numerous procedures used. The resulting data are clearly stated and nicely illustrated in the accompanying figures, leading to the conclusion that peri-acute intraspinal grafting of HSSC can represent an effective therapy which ameliorates motor and sensory deficits after spinal cord injury. I would place some caution here, as the used spinal cord compression model will have some inherent spontaneous recovery (the authors try to elucidate this by well designed control groups). It should furthermore be noted that the authors primarily demonstrate impact on spasticity recovery and termal-tactile stimulation evoked responses which may be explained by the demonstrated formation of newly formed GABAergic synapses locally in the spinal cord. However, the data do not demonstrate neither do they claim to do so that original peripheral neurons or long ascending/descending tracts in the spinal cord are restored. I would furthermore like to add that the demonstrated neuroplastic changes after spinal grafting not necessarily may be beneficial, but in humans potentially could result in neurogenic pain. Furthermore, is the cavity filling by the grafted cells impressive but also a bit disturbing as further uncontrolled growth may result in local compression and neoplasia.

The stated concerns do, however, not disclose that this paper is based on rigorous scientific testing and methods at the highest international level, and thus adds valuable new information to the field of neural transplantation in spinal cord injury.

Chapter 5 (paper V)

The fifth paper (in chapter V) with the title "Effective long-term immunosuppression in rats by subcutaneously implanted sustained-release tacrolimus pellet: effect on spinally grafted human neural precursors survival" is under revision for publication at the international renowned journal Experimental Neurology with Marian Hruska-Plochan as 8th author.

The study examines by rigorous clinical observation, and biochemical methods including chemiluminescent microparticle immunoassay, HPLC and immunohistochemistry quantitative methods including flow cytometri the pharmacokinetics of four different subcutaneously delivered/implanted tacrolimus formulations leading to the identification of biodegradable 3-months lasting pellets as the most optimal vehicle. This formulation is then used in three different concentrations in xenogenic cell grafting experiments in a rat model of ALS and a rat model of spinal cord injury (the ones used in paper III and IV), resulting in consistent presence of implanted human neurons with minimal or no local T-cell infiltration. The authors conclude that the pellet formulation represent a simple to use, effective and safe long-lasting immunosuppressive drug delivery system. This paper is based on adequate scientific testing and methods at the highest international level, although the blood sample regimes are a bit different between the four formulations and thus indicate some a priori assumptions. Likewise one may add that the 7-15 day period to reach adequate plasma levels after pellet implantation may hamper future clinical usability in spinal cord injury and the short window between the ineffective 1.9 mg dose regime and the toxic 5.4 mg dose regime also could be problematic. These concerns are, however, well discussed. I find therefore that this paper likewise adds valuable new information to the field of xenogenic and allogenic neural transplantation which furthermore is substantiated by the commercialisation of the identified pellet formulation.

Chapter 6

In the general discussion of the thesis Marian Hruska-Plochan clearly places the scientific value of the present thesis work into the scientific context of previously known data. The results of paper I-II are placed in a relevant scientific context based on our existing knowledge of HD, transgene HD models, and the possible pathogenetic role of UCHL1 in HD disease and in the proven fertility problems of the transgene minipig. Although the animal model needs to develop neurologic symptoms and the precise role of the UP system in HD and other neurodegenerative disorders remains to be further established in the future.

The results of papers III-V are likewise adequately presented as necessary further steps to develop future CNS restoration therapies and are appropriately discussed with respect to prior data. Probable difficulties in the future translation to the human clinic could be considered more, but in general, differences with other data which are expected to arise in a field are appropriately addressed, and also the final conclusions are appropriate.

Chapter 7

The references are presented in a systematic way, and are well selected covering original background work and the newest (2013) findings in the field.

Conclusion

Overall, the present thesis comprises a large and comprehensive series of experiments with regard to either the development of a transgene HD minipig and the role of UCHL1 e.g. the UP system in HD (papers I-II), and the potential role of grafting of human neural stem cells in degenerative or traumatic spinal cord disease (papers III-V). To perform all of these studies Marian Hruska-Plochan has worked with a large number of different techniques including transgenesis, animal breeding and handling, intra-spinal grafting with impressive few complications, in vivo clinical testing with observations, motor and sensory tests, electrophysiology and post-mortem analysis. The post-mortem techniques include histological processing immunohistochemical staining and analysis at the LM and EM level, high pressure liquid chromatography, flow cytometri and Time-resolved Förster Resonance Energy Transfer (TR-FRET) qualitative analysis.

Thus, even-though Marian Hruska-Plochan only has one first authorship on a submitted manuscript, and a joint first authorship on a manuscript published in a newly established

journal, and are listed as 8th author on papers III-V, it would be impossible to image how this overwhelming amount of work should be handled by a sole first author. Thus, the presented studies have their place and necessity in the scientific field and community, in that they give further understanding on HD and xenografting to the spinal cord. The thesis of Marian Hruska-Plochan has in that context served it purpose and pawed the way for many future studies. In summary, there is no doubt that this is a very good thesis work that fulfils the requirement for the award of a PhD degree.

Carsten Reidies Bjarkam MD PhD