University of Veterinary Medicine Hannover

The Libechov Minipig as a Transgenic Animal Model for preclinical research in Huntington’s Disease – development of a Phenotyping Battery including cognitive, motor and behavioral assessments

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Diese Arbeit ist meiner Familie gewidmet.

This work is dedicated to my family.
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1. Introduction

Huntington’s Disease (HD) is an autosomal-dominant neurodegenerative disorder characterized by motor, cognitive and behavioral symptoms (Walker 2007; Ross et al. 2014). Choreatic movements, dementia and depression are core manifestations in HD. It is caused by a Cytosin-Adenin-Guanin (CAG) triplet repeat expansion ≥ 36 in the Huntington gene (Huntington Study Group 1996) that translates to a misfolded mutant Huntingtin (mHTT) protein. The mHTT protein leads to neuronal dysfunction and death in wide areas of the brain including the cerebral cortex, white matter and striatum (MacDonald et al. 1993; The Huntington’s Disease Collaborative Research Group 1993; Tabrizi et al. 2009; Tabrizi et al. 2013). The progressive brain atrophy starts many years before the first symptoms of HD can be detected (Ross et al. 2014). The age of onset depends on the length of the CAG triplet repeat with longer expansions causing earlier disease onset. The Unified Huntington’s Disease Rating Scale (UHDRS) is a clinical scale to assess symptom status and progress in human HD (Huntington Study Group 1996). Motor symptoms, deficits in cognitive (Paulsen 2011; Stout et al. 2012; Bonner-Jackson et al. 2013; Paulsen et al. 2014) and behavioral domains (Craufurd et al. 2001; Fisher et al. 2014) are assessed in this scale.

Because of the monogenetic background of HD a number of transgenic (tg) and knock-in animal models were developed. The models aim to study the pathology, safety and efficacy of new therapeutic approaches in HD (Chang et al. 2015). Established animal models are e.g. nematodes, drosophila, mice, rats, sheep, monkeys and minipigs (Morton & Howland 2013; Pouladi et al. 2013). To date, especially rodent models have been used for preclinical research in HD (Crook & Housman 2011; Kim et al. 2011). Some aspects favor rodents over large animal models, e.g. generation time, litter size, housing, handling, and costs. However, despite numerous evidence in preclinical research for disease modifying effects, none of these could successfully be translated into humans yet (Venuto et al. 2012). Large animal models promise a higher probability for successful translation into the
Introduction

The Research Center PIGMOD & Institute of Animal Physiology and Genetics, Academy of Science of Czech Republic, Libechov, Czech Republic, has established the tgHD Libechov minipig (Baxa et al. 2013) with stable transmission of the HD mutation across several generations. The model was generated by lentiviral transduction and expresses an N-terminal truncated form of human huntingtin with 124 CAG/CAA repeats on chromosome 1.

17 Transgenic and 19 wildtype (wt) female Libechov minipigs (n=36 total) were housed in the central animal facility of the University of Muenster, Germany, to develop and establish a novel assessment battery with several motor, cognitive and behavioral tests targeting the characterization of tgHD minipigs inspired by the UHDRS. The minipigs arrived in Muenster in six groups (wt and tgHD mixed) of six animals each at the age of three month. Each group was housed in a temperature and humidity controlled stable with a target value of 22 degrees and 50–60% humidity. The stables had a size of 2 m² per animal and were enriched with toys, litter and hay. A daily veterinary care was provided. The animals’ weight was monitored weekly (range: 40–120 kg). The minipigs obtained regular parasite prophylaxis and hoof trimming.

Each group received a short phase of anti-panic treatment initially. After successful habituation in the new environment the minipigs were thought to follow a target stick
by using classical and operant conditioning to ensure a comfortable handling. Afterwards the battery of phenotyping assessments was initiated.

The battery included several motor, cognitive and behavioral tests in a pre-defined, controlled setting (Ott et al. 2014; Schramke et al. 2014; Schuldenzucker et al. 2014; Wirsig et al. 2014; Schramke et al. 2015). The gait test using the GAITRite® (automated acquisition system), the Hurdle Test, the Tongue Test, the Discrimination Test, the Startbox back and forth Test and the Dominance Test were elements of the Phenotyping Battery. Each test was performed biannually up to an age of three years.

Furthermore, the minipigs underwent annual MRI (Magnetic Resonance Imaging) scans under general anesthesia. The scans included multiple anatomical, diffusion-weighted and spectroscopic sequences. The MRI assessments are not part of this work and are published elsewhere (Frank et al. 2014; Nagelmann et al. 2014; Schubert et al. 2014; Schubert et al. 2015).

The aim was to find assessments that facilitate a sensitive and reproducible detection of an HD phenotype in minipigs. It was hypothesized that implementation of these tests would be feasible and well-tolerated by the animals. In addition, it was hypothesized that the data collected were suitable to conduct group comparisons between tgHD and wt Libechov minipigs, and standardized enough to include them in longitudinal studies.

Minipigs were thus proposed as a model that could play an important role in preclinical research in HD (Vodicka et al. 2005; Dolezalova et al. 2014).
2. Publications

1) “The Libechov Minipig as a large animal model for preclinical research in Huntington’s disease – thoughts and perspectives”

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Abstract:

Large animal models to explore the safety and tolerability of novel therapeutic approaches for Huntington’s disease (HD) are in exploration to achieve higher translational reliability in future studies. Recently, a Libechov minipig has been established as one new transgenic (tg) large animal model for HD. We here discuss the advantages and limitations in using this model in HD with regards to breeding, housing, handling, and with respect to homology to humans and ethical considerations. A group of tgHD and wildtype (wt) female minipigs (n=36) was used to gain first evidence about abovementioned aspects. It is concluded that Libechov
Publications

minipigs may fulfill an important role to bridge the gap between rodents and non-human primates in the translation to humans.

2) “Behavioral phenotyping of minipigs transgenic for the Huntington gene”

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Abstract

Background: While several novel therapeutic approaches for HD are in development, resources to conduct clinical trials are limited. Large animal models have been
proposed to improve assessment of safety, tolerability and especially to increase translational reliability of efficacy signals obtained in preclinical studies. They may thus help to select candidates for translation to human studies. We here introduce a battery of novel tests designed to assess the motor, cognitive and behavioral phenotype of a transgenic (tg) HD minipig model. New Methods: A group of tgHD and wildtype (wt) Libechov minipigs (n=36) was available for assessment with (1) a gait test using the GAITRite® automated acquisition system, (2) a hurdle-test, (3) a tongue coordination test, (4) a color discrimination test, (5) a startbox back and forth test and (6) a dominance test. Performance of all tests and definition of measures obtained is presented. Results: Minipigs were able to learn performance of all tests. All tests were safe, well tolerated, and feasible. Exploratory between group comparisons showed no differences between groups of tgHD and wt minipigs assessed, but low variability within and between groups. Comparison with Existing Method(s): So far there are no established or validated assessments to test minipigs in the domains described. Conclusions: The data shows that the tests presented are safe, well tolerated and all measures defined can be assessed. Prospective longitudinal application of these tests is warranted to determine their test-retest reliability, sensitivity and validity in assessing motor, cognitive and behavioral features of tg and wt minipigs.
3. Discussion

The use of Libechov minipig as a large animal model for HD exhibits advantages as well as disadvantages.

Minipigs are easy to handle and to house with reasonable costs for several years (Howland & Munoz-Sanjuan 2014). The female Libechov minipigs that were included in this study lived in six mixed groups (tgHD and wt) with six animals in stables of 2 m² per minipig. They showed a constant social hierarchy. The stables were temperature and humidity controlled with a target value of 22 degrees and 50–60% humidity, and enriched with litter and toys. The toys rotated between groups every month to preserve their interest.

Beside the fact that minipigs are polyestrous, they have a fairly short gestation period (114 days) and large litter size (9-12) compared with other large animals. That enables a faster and more economical breeding. A sufficient number of genetically modified animals such as the tgHD minipigs can be created faster and with fewer founder-animals. Hence, it was possible to order only female minipigs, approximately one half tgHD and one half wt minipigs.

Because of their similarity to humans, minipigs are promising animals for preclinical research on human diseases. Pigs, including the Libechov minipigs, have a high genetic homology to humans, in general and with respect to the HTT gene. E.g., the porcine HTT gene has a genetic homology to humans of 96% (Baxa et al. 2013) while mice only have a genetic match of 91% in this gene (Kosinski et al. 1999). The average body weight of the adult, female Libechov minipigs in this study was 45–120 kg; thus, the weight of these animals is comparable to adult humans arguing for their utilization in pharmacokinetic and pharmacodynamic studies. Another similarity to humans is that minipigs showed a wide range of weights.

Furthermore, the porcine digestive system is similar to the human one. Both, minipigs and humans are omnivore and monogastric. Pharmacological studies assessing biodistribution patterns should allow translation to humans. Additionally, testing of
oral therapeutics under similar conditions with respect to drug absorption should be feasible. An important benefit, especially for preclinical research on brain diseases, is the gyrencephalic minipig brain that is similar to humans. Although the minipig brain is fairly small (90–100 g) compared to the human brain (1300–1400 g), it is much larger than rodent brains (e.g. rats 2 g).

The long lifespan of minipigs allows long-term studies in progressive disorders; another argument for using minipigs in the tgHD phenotyping study. HD and other degenerative diseases need many years to manifest clinically. A long lifespan may be important to study the progression of disease.

Another advantage of minipig models is the possibility to anesthetize and intubate these animals for examinations such as MRI. The Libechov minipigs were easy to sedate, intubate and anesthetize. Narcosis was stable and could be maintained for a long time and administered repeatedly. Minipigs could be kept in back, prone, or lateral position to perform manipulations during narcosis. The feasibility to perform assessments in vivo such as MRI, PET (Positron Emission Tomography), CSF (cerebrospinal fluid), blood collection, and stereotactically-guided delivery of drugs into the brain (Morton & Howland 2013) is an indispensable advantage of pigs.

The social acceptance for animal research decreases with an increasing similarity of the species compared to humans. Animal research in rodents is mostly tolerated whereas research in non-human primates often leads to contention with animal rights activists. Besides, the general role and handling of animal types play roles in respect to social tolerance. Farm animals such as pigs are more socially accepted as research models than animals that are frequently kept as pets (e.g., dogs).

However, the tgHD Libechov minipig does have a few limitations as an HD animal model. The genetic construct of this model only expresses a fragment of the N-terminal part of the Huntington gene. Another limitation is the CAG/CAA repeat while humans have a pure CAG repeat in the Huntington gene. Although both CAG and CAA code for the aminoacid glutamine, the difference in nuclein acids may lead to a different functioning than the pure CAG repeat. In addition, the huntingtin fragment is
expressed with the background of two porcine Huntington genes. Another disadvantage of pigs as an animal model in general is an organizational challenge: A lot of manpower and space is necessary to use pigs as an animal model.

However, the advantages outweigh the disadvantages of the tgHD Libechov minipig. Minipigs were thus proposed as a model that could play an important role in preclinical research in HD. Therefore a test battery (cognitive, motor and behavioral tests) was developed to detect a possible phenotype of these animals, and to study the progress of the disease once there is a phenotype. Furthermore the animals underwent MRI scans to observe the minipig’s brains (the imaging data are topic of other dissertations).

The tests proposed (Ott et al. 2014; Schramke et al. 2014; Schuldenzucker et al. 2014; Wirsig et al. 2014; Schramke et al. 2015) were selected to cover a wide range of phenotypical features expected to manifest in HD (Walker 2007). Choice of the tests was partly driven by availability of data from human HD studies (Tabrizi et al. 2009; Tabrizi et al. 2012; Tabrizi et al. 2013; Paulsen et al. 2014).

The classical and operant conditioning procedures at the beginning were suitable to ensure a comfortable handling. After a short phase of habituation the minipigs were motivated to work with the experimenters, and recognized and learned their tasks quickly.

The tests can be divided roughly into 3 categories: Motor tests, cognitive tests and behavioral tests. It should be noted that each category is depended on the other categories (e.g. motor tests include cognitive components, and cognitive tests include motor challenges) such as in human HD. However, the impact of other categories may be irrelevant, as long as the primary aim is to detect a phenotype and assess the progress of disease in tgHD minipigs.

The motor tests relate to motor symptoms assessed as part of the Unified Huntington’s Disease Rating Scale Total Motor Score (UHDRS-TMS), the gold standard for clinical motor assessment in human HD (Huntington Study Group 1996; Tabrizi et al. 2009).
One motor test of the test battery was the GAITRite assessment (automated gait analysis) that has successfully been used to detect gait abnormalities in clinical HD patients (Huntington Study Group 1996; Rao et al. 2008; Tabrizi et al. 2009; Bohlen et al. 2013; Tabrizi et al. 2013). It was amenable for direct translation to minipigs. An advantage of the GAITRite assessment is the automated procedure and consequently the low rater influence. Another motor test developed was the Hurdle Test, a further test to evaluate gait coordination. The Hurdle Test took place under challenging conditions similar to the tandem walking subitem in the UHDRS-TMS in humans (Huntington Study Group 1996). The Tongue Test was a motor test to measure the tongue coordination in minipigs that is also measured routinely in the UHDRS-TMS. The assessment of tongue motor coordination in humans was translated to the Q-Motor “glossomotography” assessment (Reilmann et al. 2010), which has been applied in biomarker studies (Tabrizi et al. 2009) and clinical trials (Reilmann et al. 2015).

In addition, cognitive deficits as a symptom of HD (Paulsen 2011; Stout et al. 2012; Bonner-Jackson et al. 2013; Paulsen et al. 2014) could be measured by the Discrimination Test and by the Startbox back and forth Test in minipigs as part of the Phenotyping Battery.

Behavioral abnormalities (Craufurd et al. 2001; Fisher et al. 2014) can also be detected in HD patients. The Dominance Test aimed to evaluate behavioral symptoms in minipigs.

The motor, cognitive and behavioral tests were performed biannually to detect onset and progression of disease.

The data collected to date show that implementation of all tests of the Phenotyping Battery is practicable, safe and well tolerated. Both tgHD minipigs and wt minipigs were able to learn and perform all assessments of the Phenotyping Battery including partly tests of a fairly high level of complexity. Group comparisons showed no differences between tgHD and wt minipigs up to an age of three years in cognitive, motor and behavioral tests, but low variability within and between groups.
While the Phenotyping Battery introduced here is feasible, the onset of disease in tgHD Libechov minipigs is not reached up to an age of three years. One opportunity is that the tgHD minipigs will not develop symptoms of HD. A desirable next step would be the development of a humanized knock-in minipig model of HD. The Phenotyping Battery could be used in further pig models. However, another opportunity is that HD in tgHD Libechov minipigs may develop a slowly progressive disease similar to humans. Consequently, effective therapies for transgenic pigs could imply a higher probability of successful translation into humans. It is suggested that it will be possible to detect the phenotype with the novel test battery. Also the progression of disease may be detected because of the established feasibility of constantly recurring trainings and tests twice the year.

The tests proposed have several limitations. The Phenotyping Battery is a first set of tests that was shown to be feasible to apply and manage in a fairly large cohort of minipigs. Due to time constraints and necessity of repetitive assessments, the next step will be a rigorous selection of the tests. The selected assessments need to deliver objective evidence for an HD phenotype such as Q-Motor does in humans (Reilmann et al. 2010; Reilmann et al. 2012). Furthermore, the degree of automation of tests should be increased to increase reliability and sensitivity and to save manpower, time, and subsequently costs.

However, animal models should be replaced by in vitro alternatives where possible. The number of animals should be reduced to a minimum, and if animal models are indispensable, the experimental procedures need to be refined as thorough as possible - Replacement, Reduction, and Refinement. The overall effort to reducing the number of animals applied in research to a minimum should be in our focus. Every animal we use – no matter what species – should be enriched during the whole lifespan: Breeding, transporting, housing, handling, health maintenance, methods of euthanasia or detailed consideration whether there is the opportunity to rehome or to retire the animals should be considered wherever possible.
4. Conclusion

Large animal models for HD may be needed. The development of these models is work in progress. Current animal models will not be able to answer outstanding questions about the clinical phenotype, however, may well serve the further understanding of certain pathomechanisms of HD. In general, all animal models of any disease exhibit advantages and disadvantages.

Large animal models could play an important role to close the gap between preclinical research in rodents and clinical studies in humans. They are a promising compromise between scientific needs and environmental requirements, and should contribute to higher translational reliability and sensitivity in HD and beyond. The use of tgHD minipigs as a large animal model in preclinical studies is feasible. While the Phenotyping Battery introduced here is feasible, the onset of disease in tgHD Libechov minipigs is not reached up to an age of three years. Consequently, HD in tgHD Libechov minipigs may be a slowly progressive disease such as in humans.
5. Summary

Sarah Schramke (2016)

“The Libechov Minipig as a Transgenic Animal Model for preclinical research in Huntington’s Disease – development of a Phenotyping Battery including cognitive, motor and behavioral assessments”

Huntington’s disease (HD) is an inherited neurodegenerative disorder in humans. It is caused by a Cytosin-Adenin-Guanin (CAG) triplet repeat expansion of ≥ 36 in the IT-15 gene on chromosome 4, and follows an autosomal-dominant trait. HD is characterized by motor, cognitive and behavioral symptoms. Choreatic movements, dementia and depression are core manifestations. The symptoms are caused by the mis-folded mutant protein Huntingtin (mHTT) leading to cell death particularly of neurons within the cerebral cortex, the caudate nucleus and the putamen.

To investigate the pathways of HD, several transgenic and knock-in animal models have been established. While a lot of research has been conducted with rodents and invertebrates like mice and rats, drosophila, and Caenorhabditis elegans, this work deals with transgenic minipigs. The tgHD (transgenic HD) Libechov minipig model is one of a few large animal models (sheep, monkeys) created to explore the safety and tolerability of novel therapeutic approaches for HD prior to studies in humans. Because of the high homology between humans and pigs and their similar brain structure the tgHD Libechov minipig was chosen. It was created using lentiviral transduction, and expresses an N-terminal truncated form of human huntingtin with 124 CAG/CAA repeats on chromosome 1.

This study aimed to develop a novel Phenotyping Battery that could detect symptoms in tgHD Libechov minipigs, resembling those seen in human HD. The Phenotyping Battery should be capable of assessing the motor, cognitive and behavioral phenotype of these animals. The goal for this model is to bridge the gap between preclinical research in rodents and clinical studies in humans.
Advantages and limitations of using minipigs as a large animal model were considered, especially with regards to breeding, housing, handling, and with respect to homology to humans as well as ethical considerations.

A group of 17 tgHD and 19 wildtype (wt) female minipigs (n=36) was used to gain first evidence of the tgHD minipig phenotype. The animals were available for the following motor, cognitive and behavioral assessments: A gait test using the GAITRite® (automated acquisition system), the Hurdle Test, the Tongue Test, the Discrimination Test, the Startbox back and forth Test and the Dominance Test. Each test was performed biannually. All tests were safe, well tolerated and feasible. Furthermore annual MRI scans were performed, including anatomical, spectroscopic and diffusion weighted imaging (DWI) sequences. However, the imaging data are not part of this thesis.

Group comparisons showed no differences in cognitive, motor and behavioral tests between tgHD and wt minipigs up to an age of three years. Variability of the tests was low within and between groups.
6. Zusammenfassung

Sarah Schramke (2016)

“Das Libechov Minipig als transgenes Tiermodell für präklinische Forschungsvorhaben an der Huntington’schen Krankheit – Entwicklung einer Phänotypisierungsbatterie mit kognitiven, motorischen und psychiatrischen Tests”


Eine Vielzahl an transgenen Tiermodellen und Knock-In-Tieren wurde bereits etabliert, um die HK zu erforschen. Die bisherigen präklinischen Studien wurden hauptsächlich an Nagetieren und Invertebraten durchgeführt (z.B. Mäuse, Ratten, Drosophila und Caenorhabditis elegans). Leider konnten die Ergebnisse nicht erfolgreich in die Klinik übertragen werden. Im Fokus steht nun die Entwicklung von dem Menschen ähnlichen Großtiermodellen, um die Wahrscheinlichkeit einer erfolgreichen Translation zu erhöhen.

Ziel dieser Arbeit war die Etablierung des transgenen Libechov Minipigs als Großtiermodell für die HK. Die Homologie zwischen Mensch und Minipig, besonders in Hinblick auf das Gehirn, war ausschlaggebend für die Wahl des Tiermodells. Das Minipig wird in dieser Arbeit kritisch auf die Eignung für dieses Vorhaben diskutiert mit Hauptaugenmerk auf Zucht, Haltung, Handling, Homologie zum Menschen und ethischer Vertretbarkeit.

Das transgene Libechov Minipig wurde durch lentivirale Transduktion erschaffen und exprimiert das N-terminale Ende des humanen Huntingtins mit einer Triplet-Länge von 124 CAG/CAA auf dem ersten Chromosom.


7. Publications


pp.A30–A30. Available at:
http://jnnp.bmj.com/content/85/Suppl_1/A30.2.abstract.

8. References


References


9. Index of abbreviations

CAA           Cytosin-Adenin-Adenin
CAG           Cytosin-Adenin-Guanin
CSF           cerebrospinal fluid
DWI           diffusion weighted imaging
HD            Huntington’s disease
HTT           Huntingtin
mHTT          mutant Huntingtin
MRI           Magnetic Resonance Imaging
PET           Positron Emission Tomography
tg            transgenic
TMS           Total Motor Score
UHDRS         Unified Huntington’s Disease Rating Scale
wt            wildtype
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