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**Studies on Host-Related Pathogenesis of
Herpes simplex Type-1 Encephalitis
in Rat**

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**Karolinska
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The cover page illustration has been drawn by my son Nimród Gergő Kiss
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- II. **Influence of Perineurial Cells and Toll-Like Receptors 2 and 9 on *Herpes simplex* Type 1 Entry to the Central Nervous System in a Rat Model of Encephalitis.**
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ABSTRACT

In order to explore the molecular mechanisms of *Herpes simplex* encephalitis (HSE), a severe infection of the central nervous system (CNS) caused by *Herpes simplex* type 1 virus (HSV-1); a rat model resembling the human condition was characterized in the DA (Dark Agouti) strain. After injection into the whiskers' area HSV-1 entered the CNS at the level of the brain stem via the trigeminal ganglion, subsequently spreading to the thalamus, cortex and olfactory bulb, leading to death at five days post infection (dpi). In contrast, the Piebald Virol Glaxo (PVG) strain was found to be completely resistant to disease and without signs of immunological reactions within the CNS, since HSV-1 virus did not penetrate beyond the site of inoculation. The kinetics of HSV-1 infection in the two strains was thoroughly characterized by magnetic resonance imaging, quantitative polymerase chain reaction, virus isolation in green monkey kidney cells, histology and immunohistochemistry (IHC).

Kinetics of virus propagation and primary immune reactions following HSV-1 infection were compared between the susceptible DA and the resistant PVG strain at 12 hours post-infection (hpi), 1, 2, 3 and 4 dpi. A low expression of Toll-like receptors 2 and 9 and slower recruitment of macrophages was associated with viral replication in the perineurial cell layer and consecutive propagation to the CNS in the DA rats, while virus spread was confined to the epineurium of the peripheral nerve in the resistant PVG strain.

The underlying genetic mechanisms for the difference in susceptibility between the two strains were dissected in a F2 (DAXPVG) intercross, with genome-wide microsatellite-based genotyping. Linkage analysis revealed a very strong quantitative trait locus (QTL) on chromosome 4 regulating susceptibility to HSE. Fine mapping of the QTL by infection of additional rats with recombinations in the region, haplotype mapping of disease susceptibility in a panel of inbred rat strains, infection of congenic strains, sequencing and mRNA expression studies of the genes in the interval indicated the calcitonin receptor (*Calcr*) as the candidate gene. Functional experiments with treatment using calcitonin receptor agonists *in vivo* provided further support of the candidate gene status of *Calcr*.

Additional genetic determinants of susceptibility to HSE were studied in two other rat strains: Spontaneously Hypertensive Rat (SHR) and Brown Norway (BN), which are susceptible and resistant, respectively, to HSE, as well as in 29 BNxSHR recombinant inbred lines (RIL). The use of an already existing database of single nucleotide polymorphisms (SNPs) differing between SHR and BN revealed another significant QTL on chromosome 4 regulating susceptibility to HSE. Further analysis of the QTL using immunohistopathology indicated the Von Willebrand Factor homologue (*Vwf*) gene, which has a role in blood-brain-barrier homeostasis, as a possible candidate for regulating differences in susceptibility between the BN and SHR strains.

In summary, the present study has demonstrated a strong genetic influence on the susceptibility to HSE in a rat model that displays many similarities to the corresponding human condition. Further genetic and functional studies are needed to confirm the candidate gene status of *Calcr* and *Vwf* regulating HSE and these may ultimately lead to more effective treatments of this severe CNS infection.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACI	August Copenhagen Irish
AD	Alzheimer's disease
AIL	Advanced intercross line
BB	Bio Breeding
BN	Brown Norway
CalcR	Calcitonin receptor
<i>Calcr</i>	Calcitonin receptor gene
CD	Cluster of differentiation
CD11b/c	Complement receptor 3
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dark Agouti
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dpi	Days post-infection
E3	Fawn-Hooded
ED1	Cellular marker for the phago-lysosomal membrane
ELISA	Enzyme-linked immunosorbent assay
F344	Fisher 344
<i>Gapdh</i>	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GMK	Green Monkey Kidney
gp	glycoprotein
GWAS	Genome-wide association study
hpi	Hours post-infection
HSE	<i>Herpes simplex</i> encephalitis
<i>Hse1, 2 ... n</i>	Quantitative trait locus regulating incidence of <i>Herpes simplex</i> encephalitis
HSV	<i>Herpes simplex</i> virus
HTX-Eos	Hematoxiline-eosine
IFN, <i>Ifn</i>	Interferon
IHC	Immunohistochemistry
IL, <i>Il</i>	Interleukin
<i>i.p.</i>	Intraperitoneal, directly into the abdominal cavity

IRF, <i>Irf</i>	Interferon regulatory factor
LAT	Latency associated transcript
LEW	Lewis
LOD	Logarithm of odds
mAb	Monoclonal antibody
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MYD88, <i>Myd88</i>	Myeloid differentiation primary response factor
NFκB, <i>Nfkb</i>	Nuclear factor kappa B
NK	Natural killer
O4	Oligodendrocyte marker
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFU	Plaque forming unit
PVG	Piebald Virol Glaxo
qRT-PCR	Quantitative real time polymerase chain reaction
RARE	Rapid acquisition with relaxation enhancement
QTL	Quantitative trait locus/loci
RIL	Recombinant inbred line
<i>s.c.</i>	Subcutaneous
SHR	Spontaneously Hypertensive Rat
SNP	Single nucleotide polymorphism
TLR, <i>Tlr</i>	Toll-like receptor
TNF, <i>Tnf</i>	Tumor necrosis factor
Tuj1	Mouse monoclonal anti-neuronal class III β-tubulin
TUNEL	Transferase-mediated dUTP nick-end mediated labeling
UTP	Uridine-5'-triphosphate
VNTR	Variable number of tandem repeats
Vwf	Von Willebrand factor homologue protein
<i>Vwf</i>	Von Willebrand factor gene
WF	Wistar Furth

1 INTRODUCTION

Herpes simplex encephalitis (HSE) is a relatively rare disease manifestation, although *Herpes simplex* virus (HSV) infections are ubiquitous all over the world. HSE is, however, the most common cause of non-epidemic, acute fatal encephalitis in the western world. To study it from pathogenesis to treatment demands reliable animal models. Recent studies show that unique, monogenic divergences rather than a general immunodeficiency can predispose people to rare infectious diseases. The present study comprises the characterization of a suitable animal model for the *Herpes simplex* type 1 encephalitis and studies on the genetic regulation of the HSE pathogenesis and the early immune reactions related to it.

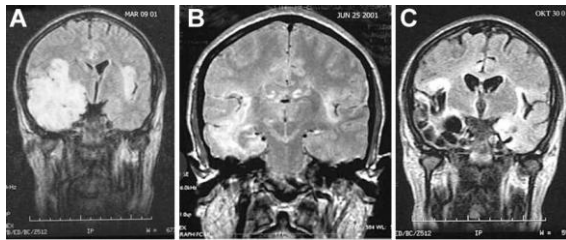
1.1 THE DISEASE

HSE is mostly caused by *Herpes simplex* virus type-1 virus (HSV-1) and in less than 10 percent by *Herpes simplex* virus type-2 (Aurelius, Forsgren et al. 1993; Aurelius, Andersson et al. 1994). HSE is a rare disease, diagnosed in 2-3 individuals per million per year (Hjalmarsson, Blomqvist et al. 2007; Sancho-Shimizu, Zhang et al. 2007; Hjalmarsson, Granath et al. 2009). Before the introduction of the antiviral drugs, or without treatment, more than 70 % of the HSE affected died and most of the survivors had severe sequels.

HSE may result from a primary, but more commonly from an earlier, reactivated HSV-1 infection. The age distribution of HSE differs from that of primary HSV-1 infection (Hjalmarsson, Blomqvist et al. 2007).

Patients present with changed behavior, altered state of consciousness, with neuro-radiological examinations revealing a focal inflammatory area in the medial part of the fronto-temporal lobe, usually unilaterally. Cerebrospinal fluid (CSF) findings include pleocytosis with predominantly lymphoid cells and increased number of red blood

cells. qPCR of the viral DNA from the CSF is the gold standard for diagnosis and should be performed at the first symptoms which point towards encephalitis.



MRI pictures of an HSE patient hospitalized in March (A), after treatment in June (B) and consequences of the encephalitis in October (C)

Herpes simplex encephalitis is an acute inflammation characterized by edema and haemorrhage, most prominently in the temporal lobes, occurring most often asymmetrically in the adult patients (Boos and M.M. 1986) with the frequent involvement of the adjacent limbic system and the meninges

overlying the temporal lobe being congested. The areas involved proceed to necrosis and liquefaction. At the earliest stage of the disease the histological changes are not dramatic and might not be specific for the condition. There is an evident congestion of the small vessels and of the capillaries in the cortex and the subcortical white matter with haemorrhagic necrosis (Booss and Kim 1984) and perivascular cuffing prominently in the second and third week of infection. Microscopic examination reveals that the involvement extends beyond the area that looks macroscopically abnormal.

In untreated patients mortality caused by HSE is over 70 % and only 2.5 % of the survivors regain normal neurological function. HSE is treated by administration of Acyclovir administered intravenously. Even with appropriate administration of antiviral therapy, there is substantial mortality and morbidity (Sköldenberg, Forsgren et al. 1984; Sköldenberg 1991; Studahl, Rosengren et al. 2000; Sköldenberg, Aurelius et al. 2006) with 19 % of the treated patients dying and 62 % of the survivors retaining some forms of neurologic sequelae (Whitley, Alford et al. 1986).

1.2 THE VIRUS

Herpes simplex virus type 1 is a double-stranded DNA virus, belonging to the family of *Herpesviridae*, subfamily of *Alphaherpesvirinae*, genus of *Herpes* viruses. The virion consists of a large double-stranded linear DNA genome encased in an icosahedral

protein capsid, which is wrapped into an envelope composed of lipid bilayer with binding glycoprotein molecules integrated in it, connected to the capsid by the tegument.

The infection of a host cell requires the binding of viral particles, *i.e.* glycoproteins on the envelope, to specific types of receptor molecules on the cell surface. The virion is thus internalised and dismantled, its capsid with the DNA migrates to the nucleus of the cell, the DNA being released into the cell nucleus, where replication and transcription of the viral genes take place. Entry of HSV-1 into the host cell involves interactions of several glycoproteins on the surface of the virus, with receptors on the surface of the host cell. The envelope covering the virus particle, when bound to specific receptors on the cell surface, will fuse with the host cell membrane and create an opening, or *pore*, through which the virus enters the host cell (Subramanian and Geraghty 2007). Viral envelope glycoprotein C (gC) binds to the cell surface heparan sulfate (WuDunn and Spear 1989; Spear, Shieh et al. 1992), glycoprotein D (gD) to at least one of the following cell receptor structures: Herpes virus entry mediator (HVEM), nectin-1 or 3-O sulfated heparan sulfate (Cocchi, Fusco et al. 2004). Glycoprotein B interacts with glycosaminoglycans on the surface of the host cell. Once bound to the cell surface gD changes its conformation and interacts with viral glycoproteins H (gH) and L (gL), which form a complex. Afterwards, gB interaction with the gH/gL complex creates an entry pore for the dismantled viral capsid and some tegument proteins. These travel to the cell nucleus by the microtubules. The tegument proteins have role both in catalyzation of the transport process and in the uptake of the viral DNA into the cell nucleus through the nuclear pore.

The infection can be asymptomatic when a small number of viral genes called latency associated transcript (LAT) accumulate, but the responsible mechanisms are largely unknown.

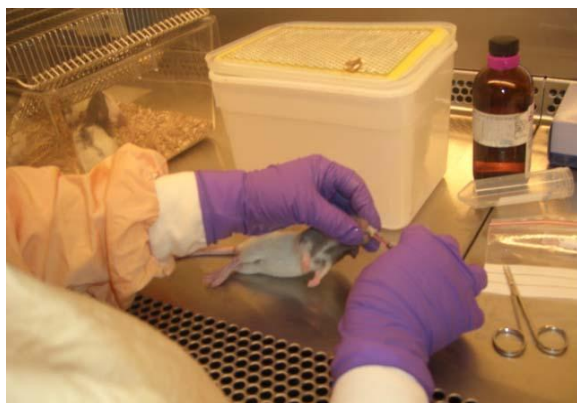
In case of recurrent herpetic disease reactivation of latent viruses has been implicated. The transcription of LAT turns to the transcription of multiple lytic genes following activation, consequently replication and virus production are enhanced, which may lead to cell death.

HSV-1, as all other *Herpes* viruses, is nuclear-replicating. In the infected cells the viral DNA is transcribed to RNA in the nucleus.

1.3 HSE MODELS

The creation of a proper animal model for HSE is a major requirement for the possibility of studying the disease from early pathogenesis, immunological reaction, outcome, treatment, relapse etc.

There have been many attempts to create a representative model for the human disease in rodents. However, most methods require a physical penetration of the blood-brain or blood-nerve barrier such as with intraparenchymal (Engel, Zhang et al. 2000) or intracerebroventricular (Ben-Hur, Itzik et al. 2004; Weidenfeld, Itzik et al. 2005) injection of the virus, injection into the vagus nerve (Thompson, Blessing et al. 2000) or injection of the HSV-1 into the eye (Cleator, Klapper et al. 1987). A newer, non-invasive method is nasal instillation or dropping into the eye (without scarring); however, these routes of administration target other cranial nerves, the olfactory nerve or the optic nerve, not the trigeminus as in humans (Fujii, Akaike et al. 1999; Garssen, van der Molen et al. 2000; Solbrig, Adrian et al. 2006; Guo, Verdrengh et al. 2009).



Injection of HSV-1 into the whiskers' pad unilaterally under Isoflurane anaesthesia

We have created a novel rat model which displays many features common with human HSE. Thus, after subcutaneous inoculation into the whiskers' area the virus enters the trigeminal nerve and via the trigeminal ganglia penetrates the brain stem unilaterally on the ipsilateral side, propagating then in a contralateral and rostral direction. The probable cause of death in

infected rats is likely to be the compromised brain stem functions.

Notably, brain stem encephalitis has been described also in human subjects, with symptoms similar to the rats including progressive quadriplegia (Jereb, Lainscak et al. 2005; Yoshidome, Hayashi et al. 2005; Miura, Kurita et al. 2009; Livorsi, Anderson et al. 2010).

2 AIMS OF THE STUDY

The aims of the project have been to:

- establish a model for HSE in the adult rat that as closely as possible mimics the human disease;
- characterize the kinetics of the infection and immune activation;
- study the degree of genetic influence on susceptibility to HSE in inbred rat strains, thereby shedding light on mechanisms of pathogenesis and finding new therapeutic targets.

3 CREATING A MODEL OF SUSCEPTIBILITY TO AND ONE FOR RESISTANCE AGAINST HSE

In order to study basic mechanisms underlying susceptibility there was a need for creating a proper animal model. The ones described in the literature differ in many ways from the clinical condition. As most adult laboratory animals are resistant to HSV-1 infection, until now a row of different more or less invasive and drastic techniques have been used to infect the rodent CNS with HSV-1, such as injecting the virus into the vagus nerve (Thompson, Blessing et al. 2000), injecting it into the *corpus vitraeum* in the eye (Matsushima, Uyama et al. 1995), intraperitoneal (Fischer, Balk et al. 1976), by intraparenchymal (Engel, Zhang et al. 2000) or intracerebroventricular (Ben-Hur, Itzik et al. 2004; Weidenfeld, Itzik et al. 2005) injection etc.

A recent, non-invasive method used by several groups is the nasal instillation of the virus into one (Jennische, Bergstrom et al. 2008) or both (Doorduyn, Klein et al. 2010) nostrils or by drops into the eyes (Solbrig, Adrian et al. 2006). These methods are less invasive than the previous methods, but unlike human HSE they target the olfactory or the optical, instead of the trigeminal nerve. In order to imitate the human disease we sought to develop a reproducible method of injecting the virus unilaterally, on the right side into the whiskers' area, targeting in this way the innervation area of the trigeminal nerve. This method would then allow for the study of pathogenic mechanisms for penetration of the virus into the nerve endings, the nerve fascicles and its further spread into the CNS, thereby giving a possibility of finding regulated gateways relating to HSE susceptibility.

3.1 VIRUS STRAIN

In order to create a proper model reflecting the features of the human disease virus strains with high infectivity and invasiveness in human patients were initially chosen.

The virus strain “F” originating from Professor Roizman’s laboratory, did not give clinical symptoms in the infected rats.

We therefore proceeded to infect the rats with the highly neurovirulent I-2762 strain. This HSV-1 strain was isolated from a brain biopsy taken from a male patient on day 2 after the onset of the first clinical symptoms of HSE. The patient died 2 days later as a consequence of the encephalitis (Sköldenberg, Forsgren et al. 1984). The virus was propagated in green monkey kidney (GMK-AH1) cells for a maximum two passages and was aliquoted and stored at -80° C. The isolate was typed as HSV-1 by enzyme-linked immunosorbent assay (ELISA) using type-specific monoclonal antibodies (mAbs) and infectivity titers were expressed in PFU/ml (Bergström and Lycke 1990). In previous experimental studies this strain showed a high degree of neurovirulence and neuroinvasiveness in both *in vivo* and *in vitro* (Bergström, Alestig et al. 1990; Bergström and Lycke 1990).

3.2 LAB ANIMAL SPECIES

Rabbits have long been used as animal models of human herpetic infections including encephalitis and have provided useful information on pathogenesis, prevention and therapy (Stanberry 1992).

Studies of mouse models of HSE dating back to the sixties have significantly improved our knowledge of HSV-1 infection and HSE pathogenesis. Murine models of HSE have shown differences in susceptibility in diverse strains of mice (Lopez 1975; Kastrukoff, Lau et al. 1986; Ellison, Yang et al. 2000) and the influence of the age on susceptibility (Johnson 1964). Subsequent findings suggested that the natural resistance of the C57BL/6 strain compared to the susceptibility of the BALB/C strain might be conveyed by an enhanced interferon α/β response, however, only providing a transient delay in HSV-1 infection (Halford, Balliet et al. 2004). Attention has been lately returning to rats after the explosion of murine models in the golden era of development of the transgenic technologies in mice in the last decades.

Rats are commonly used models in neuroscience and pharmacology. Nevertheless establishing a proper model for HSE in rats has been attempted in outbred rats, such as

Sprague-Dawley (Engel, Zhang et al. 2000) or in Wistar (Müller, Maharaj et al. 2005). One single study investigating ocular herpes has described strain differences in susceptibility towards HSV infection (Nicholls, Benylles et al. 1994), but this line of research was not pursued beyond the initial observation.

3.3 LAB ANIMAL STRAINS

In order to reduce experimental variability we decided from the start to work only with inbred rat strains established by at least 20 generations by brother-sister mating, the individuals of which are homozygous at all loci. As inbred Dark Agouti (DA) and Piebald Virol Glaxo (PVG) rats have been shown earlier to differ in their vulnerability to autoimmune neuroinflammation and their response to nerve injury (Holmdahl, Olsson et al. 1985; Lorentzen, Andersson et al. 1997; Weissert, Wallström et al. 1998; Lundberg, Lidman et al. 2001; Lidman, Swanberg et al. 2003; Abel, Plancoulaine et al. 2010) these two strains were chosen initially.



The susceptible DA and the resistant PVG rat

A first set of experiments demonstrated susceptibility in DA and resistance in PVG rats and further testing was subsequently performed in an extended set of inbred rat strains, with the following being classified as

susceptible: Lewis (LEW), Fisher 344 (F344), Spontaneously Hypertensive Rat (SHR) and Goto-Kakizaki type 2 diabetic rats (GK) with a similar disease phenotype as DA, and as resistant: Bio Breeding type 1 diabetic rats (BB) and Brown Norway (BN), having a similar resistance phenotype as PVG. Meanwhile other inbred strains were identified having intermediate phenotype, *i.e.* some individuals developed encephalitis while others presented only mild or no symptoms of disease at all, such as the August Copenhagen Irish (ACI), Wistar Furth (WF) and Fawn-Hooded (E3) rats.

3.4 SPECIAL LAB ANIMAL POPULATIONS

To confirm or to exclude the influence of certain genes or chromosomal regions congenic populations can be used. The congenic lines are genetically identical to the background strain except for the gene or the genomic region which has been transferred by generations of breeding and backcrossing from another inbred strain. Thus, shortly after the establishment of the model we tested if the susceptibility/resistance in the DA and the PVG rat strains was conveyed by the MHC genes by infecting reciprocal congenics: DA rats carrying the MHC genes of the PVG rats (Dark Agouti-*RTI^c* DA) or a genetic fragment containing the *Mhc2ta* gene on chromosome 10 (DAc10PVG) and PVG rats carrying the MHC genes of the DA rats (Piebald Viral Glaxo-*RTI^{av1}* – PVG.1AV1, shortly referred to as PVG.A) and a reciprocal congenic of the chromosome 10 fragment (PVGc10DA) (Weissert, Wallström et al. 1998; Swanberg, Lidman et al. 2005).

These experiments showed that neither the MHC complex nor the chromosome 10 fragment regulated disease susceptibility. Since the PVG.A strain was as resistant to HSE development as the PVG strain, it was used in all further experiments.

3.5 LAB ANIMAL AGE

Age of the rats at the time point of the infection plays a crucial role in their susceptibility vs. resistance to develop HSE. Our model has been established in young adult animals. All DA rats infected with HSV-1 at the age of 45 days developed lethal HSE, while none of the PVG rats of the same age presented any clinical symptoms of disease. However, interestingly, 25 days old PVG rats were susceptible to HSE, while 65 days old DA rats became resistant to disease development. The developmental aspect of susceptibility to HSE, most likely related to the development of some components of the innate immune responses has been described already in the sixties (Johnson 1964).

In order to reduce a source of variability and to ensure reliability of data we infected the rats in all our experiments at the age of exactly 45 days.

3.6 ROUTE OF INFECTION

In order to mimic as confidently as possible the route of HSV-1 propagation in patients and also to avoid invasive infection techniques we have chosen to inject the virus subcutaneously into the whiskers' area, corresponding to the labio-facial area in humans most commonly affected by the virus.

After being thawed to room temperature HSV-1 has been injected instantaneously *s.c.* into the area of the whiskers' base on the right side unilaterally under 2 % Isoflurane anesthesia.

The method enabled with great accuracy to compare the viral presence between the ipsi- and the contralateral side in the different anatomical compartments and to follow the chain of events from the uptake and propagation of virus from the infection site through the trigeminal ganglion on the ipsilateral side to the trigeminal nucleus in the brain stem, and the further spread of HSV-1 into the brain stem ipsilaterally, towards the contralateral side and its propagation in caudo-rostral direction to the thalamus, first to the contralateral, then to the ipsilateral side. One of the most interesting findings in this study was the completely different distribution of HSV-1, immunological cells and receptor expression in the different anatomical compartments (epi-, peri- and endoneurium) belonging to the peripheral nerves observed in DA vs. PVG rats.

3.7 DOSE

The use of the proper dose for infection has also shown to be of great importance. DA rats infected with doses as low as 10^6 PFU HSV-1 did not develop HSE, while doses higher than 3×10^6 seemed to increase survival, illustrating an extremely narrow, traditionally of virology point of view almost ignorable interval of doses, which most efficiently resulted in lethal encephalitis. Interestingly, in pilot experiments DA rats infected with higher doses developed clinical signs of encephalitis at day 5, then after three-four days started to recover and regained weight, but after a couple of weeks fell sick and died in a relapse. This observation would be a very interesting starting point in

the future for a study focused on a more chronic, relapsing form of HSE (Sköldenberg, Aurelius et al. 2006).

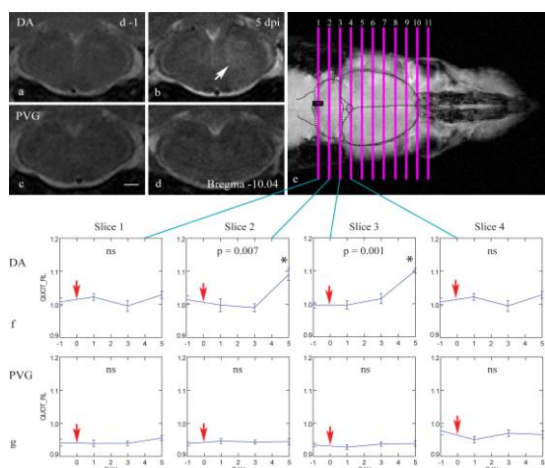
3.8 GENDER

We have tested the model in both male and female rats and could show that there were no discernible differences in infectivity or disease outcome between the two genders. Thus, in most experiments, except for the genetic study we have used male rats in order to exclude variability which could originate in body weight differences or the changing hormonal levels and behavior depending on the estrus cycle in females.

4 VALIDATION OF THE MODEL

Excluding all possible sources of variability and establishing a reliable and accurately reproducible model for both susceptibility and resistance we proceeded to their validation (Bereczky-Veress, Lidman et al. 2008).

4.1 MAGNETIC RESONANCE IMAGING



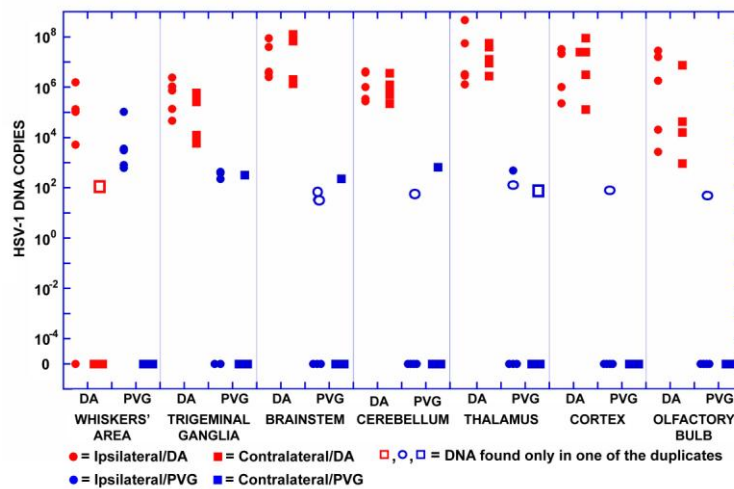
Magnetic resonance image of the brain stem showing oedema in DA rats compared to PVG

Non-invasive imaging methods give the possibility to follow the kinetics of the inflammation from the healthy, uninfected animal to the fully developed clinical disease *in vivo*. The T2-weighted RARE magnetic resonance imaging method is most commonly used for the detection of the brain pathogenesis caused by encephalitis and other similar conditions (Hennig, Nauerth et al. 1986). The method

is based on measurements of water content, detecting changes in magnetic resonance properties of the tissues with

edema caused by the inflammation compared to the healthy ones and transforms the information into images. We could visualize by this method that the encephalitis started in DA rats first in the brain stem at the level of the trigeminal nuclei on the ipsilateral side while PVG rats did not show signs of pathological changes of the brain at any time point.

4.2 QUANTITATIVE POLYMERASE CHAIN REACTION

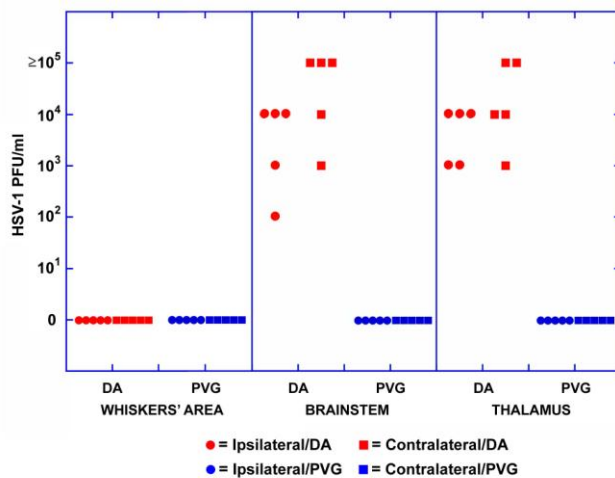


Significantly higher HSV-1 DNA copy numbers in the trigeminal ganglia and brain compartments of the DA rats

and five compartments of the brain, as brain stem, cerebellum, thalamus, cortex and the olfactory bulb (Namvar, Olofsson et al. 2005). In all compartments studied, except for the whiskers' area, viral DNA was present in $10^3 - 10^5$ -fold higher quantity in DA rats, while in PVG rats only traces of HSV-1 DNA could be detected occasionally at 5 dpi. In the whiskers' area HSV-1 DNA could be detected only in the ipsilateral side, in the trigeminal ganglia higher levels of viral DNA was present in the ipsilateral side and in the different segments of the brain there was no difference between viral DNA levels in the ipsi- and the contralateral side. Kinetic studies have shown that HSV-1 DNA was present in high levels in the whiskers' area of both DA and PVG rats and in the trigeminal ganglia of the DA rats from 12 hpi to 5 dpi, while in the brain stem of DA rats levels increased from 2 dpi. No viral DNA was isolated from the trigeminal ganglia or the brain stem of the PVG rats.

To show distribution of viral spread from the infection site to the different compartments in the brain we performed qRT-PCR studies of the viral DNA in tissues sampled from infection site, *i.e.* the whiskers' area, the trigeminal ganglia

4.3 VIRUS ISOLATION



Polymerase chain reaction detects viral DNA presence in the compartments studied, but does not give any indication regarding infectivity of the virus. We therefore performed virus isolation in green monkey kidney cells from the tissues of the whiskers' area and of

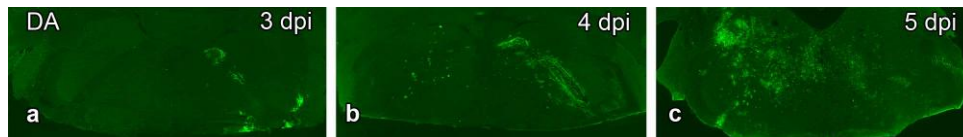
two compartments of the brain to study if living virus could be retrieved from these compartments. Interestingly, no living virus could be obtained from the whiskers' area of neither DA, nor PVG rats at 5 dpi. In contrast, high levels of virus were obtained from the brain stem and the thalamus of the DA rats at 5 dpi, while these compartments in the PVG rat remained completely free of detectable virus. Kinetic studies showed that the quantity of infective HSV-1 decreased in the whiskers' area in both strains within the first 2-3 days in parallel to an increase of replicating HSV-1 in the trigeminal ganglia and the brain stem solely in DA rats.

4.4 IMMUNOHISTOCHEMISTRY

Next, immunohistochemistry (Coons 1958; Pease 1962; Zamboni L. 1967) was used to gain a better understanding of the spread of the HSV-1 and the inflammatory process taking place in the brain. By staining different markers in tissue sections sampled from different compartments of the brain we have visualized HSV-1 penetration from the level of the trigeminal nuclei in the brain stem in rostral direction. The penetration of the HSV-1 was followed by a tissue reaction with infiltration of immunological cells. These two aspects of the encephalitis were shown by staining for activated microglia and astrocytes. OX6 is a MHC II marker expressed mainly on activated microglia and GFAP detects astrocytes. By staining of these two markers we proved that in DA rats

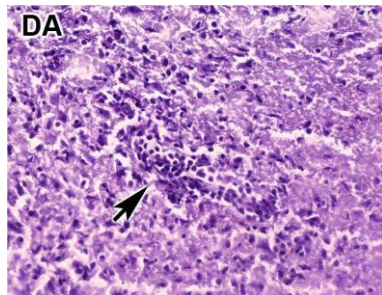
there was massive tissue activation compared to PVG rats, the brain of which remained as unaffected as that of the uninfected controls.

Immunohistochemistry has also revealed the differences in the infiltration of the immune cells into the brain of the two HSV-1-infected rat strains. We could show that ED1⁺ phagocytic cells, NK cells, CD4⁺ T cells and CD8⁺ T cells penetrated into the brain and invaded the HSV-1 infected areas while in the brains of PVG rats there was no infiltration of these cells (Dijkstra, Dopp et al. 1985).



Immunohistochemistry staining of the kinetics of HSV-1 spread in the brain stem of DA rats

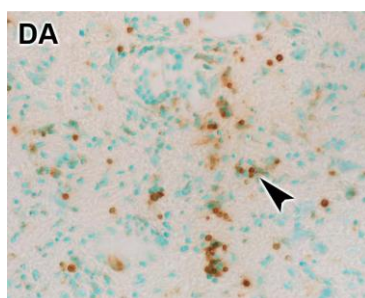
4.5 HISTOLOGY



Infiltrating inflammatory cells in the brain stem of the DA rat

The traditional histology method of Hematoxylin-Eosin staining visualizing the cell walls and the cell nuclei confirmed the conclusion drawn by immuno-histochemistry, *i.e.* severe tissue damage and many infiltrating cells were detected in the brain of the DA rats while no pathology of the brain was detected in the PVG rats.

4.6 TUNEL-STAINING

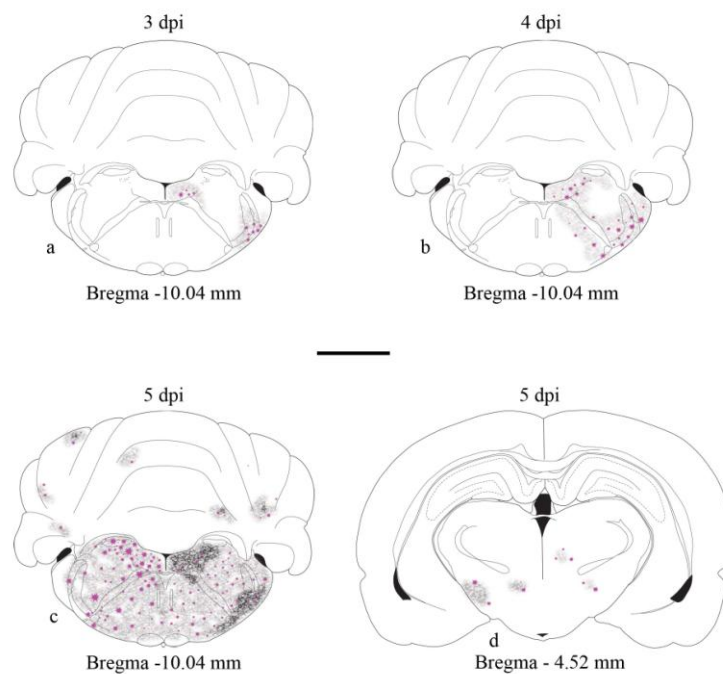


Apoptotic cells in the brain stem of the DA

Apoptosis is an important component of the virus-induced neuronal injury (Thompson 1995; Geiger, Nash et al. 1997; Griffin and Hardwick 1999; DeBiasi, Kleinschmidt-DeMasters et al. 2002; Perkins, Gyure et al. 2003; Pongpanich, Bhattarakosol et al. 2004; Huang, Lin et al. 2005;

Mori, Goshima et al. 2005; Sabri, Granath et al. 2006). The transferase-mediated dUTP nick-end labeling method is based on the labeling of the DNA strand breaks occurring in the apoptotic cells. Thus, TUNEL-staining is used to assess programmed cell death – apoptosis, an important aspect of the brain injury in HSE in the tissues. Our experiments have shown that apoptosis occurred in the HSV-1 infected brain stem of the DA rats; however no apoptotic activity was detected in the brain stem of the PVG rats.

4.7 SCHEMATIC PICTURE OF THE HSE IN THE DA RATS



Schematic picture of the kinetics of the HSV-1 penetration and the consecutive tissue reaction in the CNS of the DA rats (Paxinos and Watson 1998)

5 AIMS OF THE STUDIES BASED ON THE MODEL

Once the model of susceptibility in DA and resistance in PVG strains has been characterized we found ourselves in front of a large palette of directions we could conduct our interest and studies towards. The field of research focusing on HSE pathogenesis and treatment is huge and in spite of decades of intensive research there are many questions still unanswered.

5.1 EARLY IMMUNE REACTIONS RELATED TO HSE

Our interest focused on studies regarding the differences in the ability of the innate immune system of the susceptible and the resistant rat strains to detect the viral agent respectively to mediate and provide immediate antiviral reactions. We have explored the early kinetics of the HSV-1 infection and the consecutive host-dependent innate immune responses after 12 hours and each of the first four days post infection, demonstrating a lower and delayed ability in the susceptible DA strain to detect the virus as compared to the resistant PVG rats and that different anatomical compartments of the peripheral nerves got HSV-1 infected at the injection site in the two strains leading to the development of lethal encephalitis in the DA strain and to resistance in the PVG.

5.2 GENETICS OF HSE

In parallel to the immunological studies we started the most fascinating, but by far largest and time-demanding part of the study aimed at dissecting the genetic regulation of HSE. One of the most puzzling questions facing researchers is that why an immune competent person is affected by the disease, while others do not. By studying inbred strains, the individuals of which are genetically identical within the strain, but with large inter-strain differences, resulting in a completely different susceptibility pattern

towards a particular disease, we have the means to study the genetic involvement in the pathogenesis as these strains could be models for different human individuals.

In this project we have made an attempt to identify genes regulating susceptibility to HSE by using the inbred rat strains and different populations of rats resulting from special breeding schemes established between the two strains.

5.3 TREATMENT STUDIES

We have conducted treatment studies in DA rats in order to attempt to attenuate disease outcome. In spite of the fact that these experiments generated a large amount of interesting and relevant data, the results are not included in this thesis, since further studies are needed for final conclusions.

Preliminary results have shown that Acyclovir, currently used as a corner stone of HSE treatment in patients, remained the best treatment in the rat model too, while substances up-regulating early innate responses were not efficient, or even reduced the efficacy of Acyclovir. Interestingly, treatment with rat Interferon β , considered to have a beneficial role in HSV-1 infection (Olofsson, Holmberg et al. 2003; Halford, Balliet et al. 2004; Olofsson, Nerstedt et al. 2007) given at 1 and 3 dpi has caused death earlier when compared to untreated rats. As well, recent studies (Casrouge, Zhang et al. 2006) speculated that a deficiency in UNC-93 protein regulating intracellular TLR responses leading to reduced type I interferon production might be the genetic cause of susceptibility to HSE.

It is known that immuno-compromised patients only develop subacute encephalitis. Our findings also support the notion that it is the immune reaction, not the HSV-1 presence in the CNS *per se*, that is the main cause of the vast tissue damage leading to death in HSE. One of these findings is the worse outcome of the disease in the DA rats treated with Interferon β ; another is the presence of HSV-1 in the CNS of the clinically resistant BN rats.

5.4 STUDIES OF THE SUBCLINICAL INFECTION

Preliminary pilot studies by MRI have disclosed that chronic infection of the susceptible DA strain with subclinical dose of HSV-1 resulted in severe brain pathology in otherwise completely symptomless rats.

Since the overwhelming majority of the population is asymptomatic carrier of the HSV-1 in the CNS, I believe that the study of the aspect of the subclinical infection would have been extremely interesting and could be observed in future through long-term experiments *in vivo* by the means of MRI. Unfortunately in lack of time we could not focus our attention onto this fascinating aspect.

6 EARLY IMMUNE RESPONSES TO HSV-1 INFECTION IN HSE

Innate immunity plays a crucial role in the control of HSV-1 infection (Wuest, Austin et al. 2006; Wuest and Carr 2008). The study has attempted to clarify the differences in kinetics between the susceptible DA and the resistant PVG rats in the spread of the HSV-1, the tissue reaction and the immune cell infiltration to the virus affected compartments parallel to observations of the expression of a range of immune-related genes at the level of the infection site, the trigeminal nerve and the brain stem (Bereczky-Veress, Abdelmagid et al. 2010). The combination of the mRNA expression results obtained by qPCR at the molecular level and the immunohistochemistry findings at the cellular level offer satisfactory hints to give an insight to what might happen in the peripheral tissue after the penetration of the viral pathogen.

qPCR analysis amplifying a sequence of a target of interest has been performed on cDNA made from the RNA extracted from the tissue samples from the whiskers' area, trigeminal ganglia and the brain stem. The quantification was based on the comparison to the expression levels of commonly used housekeeping genes such as *Gapdh* and *Hprt*.

Immunohistochemistry is based on the binding of a primary antibody to a target located of the marker to be studied. The binding was visualized by the binding of an immunofluorescence-labeled secondary species-different antibody to the primary one. There are other immunohistochemistry methods which amplify the signal of the binding of the primary antibody, but these methods could not be used for the HSV-1 infected tissues, because the virus-infected tissues bound the amplification substances unspecifically.

6.1 VIRUS DETECTION IN THE INFECTED ORGANISM

Toll-like receptors are pattern recognition receptors of the innate immune system present on the surface and inside of many cell types detecting general, mostly well-conserved characteristics of different classes of pathogens, as for example double-stranded DNA or glycoproteins (Sarangi, Kim et al. 2007).

Tlr2 and *-9* play the most important role in recognizing viral envelope glycoproteins (Bsibsi, Ravid et al. 2002; Compton, Kurt-Jones et al. 2003; Krug, Luker et al. 2004; Boehme, Guerrero et al. 2006; Sato, Linehan et al. 2006; Sorensen, Reinert et al. 2008; Goethals, Ydens et al. 2010; Peltier, Simms et al. 2010), therefore we have investigated these molecules, in parallel to *Tlr3* and *-4*. Indeed, we have found significantly reduced levels of *Tlr2* and *-9* in the susceptible DA rats at early time-points after infection compared to the resistant PVG rats, which were more efficient in detecting HSV-1 and could more rapidly signal further for adequate immune reaction. The role of *Tlr2* and *-9* in the recognition of Herpes viruses has been demonstrated by *in vitro* experiments (Sato, Linehan et al. 2006). It has also been shown that these two receptors act synergistically (Sorensen, Reinert et al. 2008). The results obtained here suggest that in our double model of susceptibility vs. resistance the mRNA expression levels of these two Toll-like receptors at early time-points after infection play crucial part in allowing the viral pathogen to invade the CNS or to protect the host against the disease.

On the other hand, defects in UNC-93 protein regulating intracellular TLRs and specifically TLR3, has been recently demonstrated to have a role in susceptibility to HSE (Casrouge, Zhang et al. 2006; Brinkmann, Spooner et al. 2007; Zhang, Jouanguy et al. 2007; Akashi-Takamura and Miyake 2008). In our model, on contrary, elevated levels of *Tlr3* were detected at the infection site in the susceptible DA strain; therefore we assumed that susceptibility to HSE was not dependent on lack of *Tlr3* in our model.

6.2 THE SIGNALING

Once the cell-associated pattern recognition receptors have detected the entry of the viral pathogen a cascade of events in the Tlr pathway is started. Adapter proteins (such

as Myeloid differentiation primary response factor 88 – *Myd88*) are recruited leading to enrollment and activation of protein kinases. This is followed by activation of different transcription factors (as Nuclear factor kappa B – *Nfkb*, Interferon regulatory factor 3 and 7 – *Irf3*, *Irf7*), gene transcription and expression of proinflammatory cytokines (such as Tumor necrosis factor α – *Tnfa*, *Il6*), chemokines, endothelial adhesion molecules, costimulatory molecules and antiviral cytokines (as Interferon β – *Ifnb*).

In our study focusing on the early immune responses to HSV-1 infection we paid attention to the most important molecules involved in the signaling cascade.

6.2.1 Adapter protein *Myd88*

Comparing the mRNA expression of *Myd88* in the susceptible DA and the resistant PVG rats we did not find differences at any compartment except for a minor difference in the expression in the brain stem ($P \leq 0.05$) at 3 dpi.

In an earlier study it has been shown *Myd88* played a major role in the signaling cascade since *Myd88*^{-/-} mice were highly susceptible to lethal encephalitis (Mansur, Kroon et al. 2005).

Considering that after infection *Myd88* levels raised even in the trigeminal ganglia and the brain stem of the PVG rats, where the virus did not penetrate, we assumed that induction of this adapter protein occurs at a systemic level. In the view of the fact that there was no difference between the expression levels of this adapter protein between the DA and the PVG strains at any level, we could conclude that *Myd88* did not play a role in the susceptibility vs. resistance to HSE in our model.

6.2.2 The transcription factor *Irf7*

Analysis of mRNA expression of *Irf7* required in the *Tlr* signaling cascade (Honda, Yanai et al. 2005) revealed very high expression levels in both rat strains, with even higher levels in all anatomical compartments of DA rats ($P \leq 0.001$). Thus, we could exclude the possibility that lack of expression in *Irf7* might be the cause of the difference of susceptibility to HSE in the two rat strains. Results in mRNA expression levels of the transcription factor *Irf3* were difficult to interpret and may be a consequence of low expression levels.

6.2.3 The inflammatory cytokines *Tnfa* and *Il6*

DA rats expressed significantly higher mRNA levels of the pro-inflammatory cytokine *Tnfa* ($P \leq 0.001$) in all the anatomical compartments studied, but these levels proved to be unprotective against HSE. Looking onto the cellular level, it has been shown that *Tnfa* penetrated the perineurium (Sorkin, Xiao et al. 1997; Bove, Weissner et al. 2009). This compartment has been proven in our study to have a special role in susceptibility to HSE.

mRNA expression levels of *Il6* did not differ between the two strains at the level of the whiskers' area and were significantly higher in the trigeminal ganglia and the brain stem of the DA rats. Since HSV-1 did not penetrate into these compartments of the PVG rats we assumed that *Il6* was expressed at local level where the HSV-1 was present and was not the cause of the susceptibility to HSE.

6.2.4 The type I interferons, *Infβ*

Recently it has been shown that interferon responses are important for HSE type 1 (Jouanguy, Zhang et al. 2007; Zhang, Jouanguy et al. 2007). Similar mRNA expression levels of *Infβ* could be observed after infection in both rat strains in all anatomical compartments except for a difference in the brain stem at 4 dpi ($P \leq 0.05$), when DA rats had higher values than PVG rats, which cannot explain differences in susceptibility to HSE, since HSV-1 did not penetrate into the CNS of the PVG rats.

6.3 OBSERVATIONS AT THE CELLULAR LEVEL

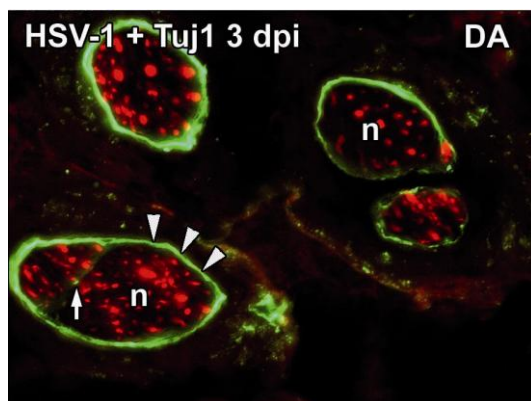
While qPCR reveals the mRNA expression of the target genes in the tissue as a whole at molecular level, immunohistochemistry enables to see the cellular compartment. Since we were aware from the validation of the model that HSV-1 did not penetrate into the trigeminal ganglia and the CNS of the PVG rats and did not bring about tissue reaction or immune cell infiltration in these compartments, our attention was focused primarily on the infection site.

6.3.1 At early time points HSV-1 infected small nerves in both strains

In tissue sections sampled from the whiskers' area at 12 hpi we detected similar spread of HSV-1, penetrating the epineurium of the nerve fascicles and in the immediate vicinity of small nerves in both strains. This might indicate that HSV-1 was present in the tissue at this time-point at the infection site, but that replication has not yet started, or still was on an initial stage in preferential cell types.

6.3.2 After 1 dpi different distribution of HSV-1 in DA than in PVG

The most exciting finding in the immunology study was the observation of the different spread of HSV-1 in the two rat strains. When validating the model we have described the kinetics of the HSV-1 in the whiskers' area. We have shown by qPCR that viral DNA was present at constantly high levels in the whiskers' area of both DA and PVG rats. By using virus isolation (virus culture) in green monkey kidney cells we stated also that infective HSV-1 was present at high levels in both DA and in PVG rats, decreasing rapidly at later time points from 10^5 to 10^1 PFU/ml in DA rats and from 10^4 to 0 PFU/ml in the PVG, but at the time of the characterization of the model had a poor understanding of what was happening at this level.



After 1 dpi HSV-1 staining had a clearly distinguishable difference in spread in the whiskers' area of DA and PVG rats. While in the PVG rats HSV-1 positivity was localized at all later time points in the outer part of the epineurium, in DA rats it spread to the perineurial layer glowing in a continuous ring-formed pattern around the

nerve fascicles, with some traces of staining even in the endoneurium. This finding might indicate that the theory of the retrograde axonal transport of the virus *vs.* the transport through the cells of the perineurial layer towards the CNS has to be re-evaluated.

6.3.3 Immune cell activation

As HSV-1 did not penetrate either the peri- or endoneurium of the peripheral nerves, nor the trigeminal ganglia or the brain stem of the PVG rats, these compartments remained shielded from tissue reaction and immune cell infiltration. However in the whiskers' area we observed by Iba1 and ED1 staining earlier phagocytosing macrophage and dendritic cell recruitment (Smirkin, Matsumoto et al. 2010) in the PVG rats than in the DA. On the other hand in DA rats there was an earlier recruitment of NK and CD8⁺ cytotoxic T-cell recruitment than in the resistant PVG strain leading also to an altered Schwann-cell morphology (Jessen and Mirsky 1984; Cheng and Zochodne 2002; Triolo, Dina et al. 2006).

6.4 CONCLUSIONS REGARDING INNATE IMMUNE RESPONSES IN HSE

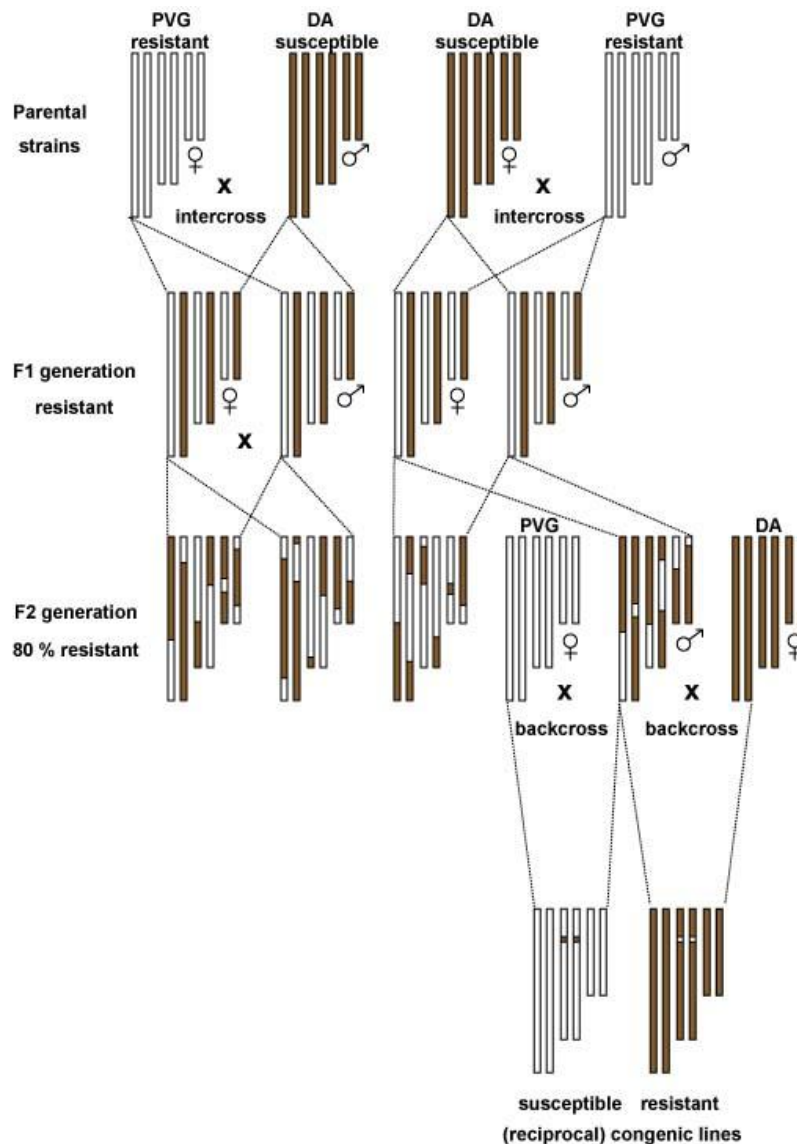
In this study we have found different patterns of host defense against HSV-1 infection in the two strains. The DA rats presented lower levels of *Tlr2* and -9 together with the earlier recruitment of NK and CD8⁺ cells and delayed engagement of macrophages as compared to the PVG rats which might indicate a weaker host defense facilitating HSV-1 entry to the peripheral nerves, replication and spread to the CNS. Nevertheless, the observation of the host strain-dependent differences in spread and replication of the HSV-1 in the perineurial cell layer might point towards the hypothesis, that the immunological differences between the two strains are more consequences of the infection and as a matter of fact the genetically determined characteristics of the perineurial cells play the decisive role in the viral entry to the CNS.

7 GENETIC STUDIES

Once establishing a model of lethal susceptibility and a model of total resistance in two inbred rat strains our interest was directed towards the genetic aspect of disease regulation.

7.1 GENETIC STUDIES IN DA AND PVG INBRED RAT STRAINS

The first step in this study was to create an F1 intercross between the susceptible DA and the resistant PVG strain and to infect them at the age of 45 days. All individuals of the F1 generation, in which the genetic contribution of each parental strain is 50 %, were resistant to HSE development after infection. By crossing individuals of F1 with each other we accomplish the mendelian segregation of the genes of the two strains in the F2 generation. Infecting F2 individuals with HSV-1 we found 20 % of the rats to be susceptible to lethal HSE, and the rest surviving the infection without any clinical signs of the disease, except for a minor drop in some individuals in the body weight development curve at around 5 dpi, which we considered as being of intermediate phenotype.



7.1.1 Microsatellite marker-based genotyping

Microsatellites are short repetitive segments of non-coding DNA named also variable number of tandem repeats (VNTRs). In a random population the numbers of the repeats in a microsatellite at a certain locus inherited from each parent differ, but in genetically distinct inbred lab animal populations, after many (at least 20) generations of brother-sister mating all individuals are genetically identical, thus the number of the repeats of the microsatellite marker at a certain locus is also identical in each individual on both alleles. In another inbred strain the number of the repeats in a microsatellite marker can be different, but with all individuals within this strain sharing the same number of repeats on both alleles.

This phenomenon of microsatellite polymorphism among different inbred strains allows for the tracing from which of the two parents genetic material has been inherited

in a F2 intercross. At a specific location this theoretically will result in 25 % homozygous for strain A, 25 % homozygous for strain B and 50 % heterozygous animals.

The method is time consuming as one must identify a sufficient number of microsatellites throughout the whole genome and each animal has to be genotyped by qPCR for every microsatellite marker. Only in the initial experiment a total of 239 F2 individuals were genotyped with 141 markers, resulting in 33,700 PCR reactions!

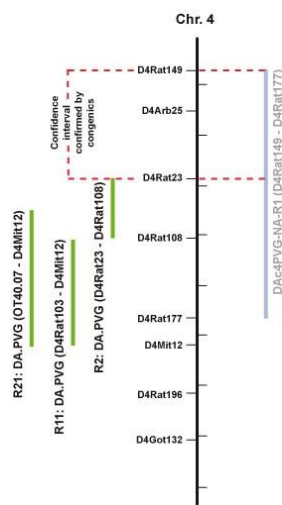
7.1.2 Linkage analysis

After crossing individuals of the F1 generation intercross we obtained an F2 cohort of 239 rats of both genders, which were all infected at the age of 45 days and followed for body weight curve and disease development. F2 males showed 20 % of disease incidence, with an onset of the disease at 6 dpi, while female rats displayed a disease incidence of 10 % and slightly later onset of the disease at 8 dpi.

A first round linkage analysis based on the 180 F2 individuals and 141 microsatellite markers calculated with the statistical software R 2.8.0 (Broman, Wu et al. 2003) identified a very strong linkage to a region on rat chromosome 4 regulating HSE incidence with a logarithm of odds/likelihood (LOD) (Laird, Zijderveld et al. 1991; Churchill and Doerge 1994; Jacob, Brown et al. 1995; Lander and Kruglyak 1995; Sen and Churchill 2001; Manichaikul, Dupuis et al. 2006) score of 29.5 and a confidence interval of 6.8 Mb. This quantitative trait locus (QTL) regulating HSE in our F2 cohort has been named *Hse1*.

Adding phenotype observations and genotyping results of the rest of the F2 rats to the initially selected ones and performing further linkage calculations we found additional QTLs on other chromosomes regulating other aspects of the HSE phenotype such as date of onset of the disease, body weight loss between 4 and 5 dpi, between d 0 and 5 dpi etc.

7.1.3 Congenic lines confirm the confidence interval



Congenic populations share the whole genome of the background strain except for a well-limited genomic region, which, by at least ten generations of microsatellite-assisted selective breeding has been transferred from a strain with a different phenotype onto the background strain (Wakeland, Morel et al. 1997). In our lab there were already existing congenic lines having PVG genomic fragments bred onto DA background, but being homozygous for DA alleles in *Hse1* (Marta, Stridh et al. 2010). We tested their susceptibility to HSE. All these

congenic lines developed disease further supporting the disease regulatory effect of *Hse1*.

7.1.4 Refining analysis: design of new microsatellite markers

Once the main QTL was identified on a whole genome level, we could focus our efforts to further map polymorphisms found within the confidence interval. Thus, new microsatellite markers were designed, with re-typing of the initial cohort of rats as well as inclusion of a new cohort of rats enriched for individuals with recombinations within the CI. These efforts together reduced the confidence interval to around 1.01 Mb.

7.1.5 Haplotype mapping

Haplotypes are DNA sequences inherited together. In our case we refer by haplotype mapping to a comparison of genomic sequences, as defined by microsatellite genotypes, across different inbred strains. By comparing the degree of susceptibility with the pattern of genetic polymorphisms it is possible to find support for a disease regulatory role of certain regions within the CI of the QTL. More clearly: if two susceptible or two resistant strains are polymorphic for a certain genomic sequence within the interval, one can most likely rule out the role of that genomic sequence and focus on those, which are not polymorphic. In contrast, if comparing two strains, one susceptible and the other resistant, the underlying genetic polymorphism is likely to be localized in the genomic region which is polymorphic.

Thus, we have compared the genomic region consisting of the 1.01 Mb CI in a panel of inbred rat strains, some of which we have found to be susceptible (Lewis, Fisher 344, Spontaneously Hypertensive Rat and Goto-Kakizaki), resistant (Bio Breeding and BN) or to have an intermediate phenotype (August Copenhagen Irish, Wistar Furth and Fawn-Hooded) towards HSE development. The haplotype map reduced the confidence interval to a suggested 0.17 Mb, but also revealed the complexity of the genetic regulation of susceptibility to HSE, since the resistant BN strain had the genotype of the susceptible and intermediate strains within this region, while the susceptible GK shared genotype of the resistant PVG.

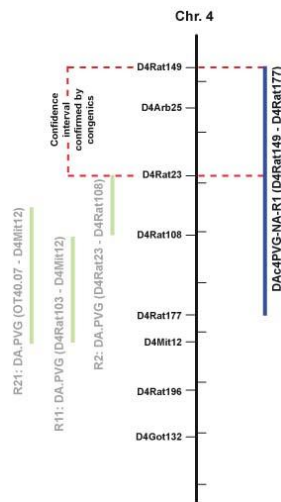
7.1.6 Positioning the candidate gene

After narrowing the confidence interval by further microsatellite markers, analysis of additional F2 rats and comparison to other inbred rat strains we proceeded to the sequencing of the genes within the CI. In this way we could rule out 6 out of the 9 remaining genes, which did not differ between the two strains.

Three remaining genes, *Ccdc132*, *Calcr* and *Tfpi2* were found to have sequence polymorphisms between the DA and the PVG strain, but these were found on untranslated intronic or 5' regions or were synonymous single nucleotide polymorphisms (SNP).

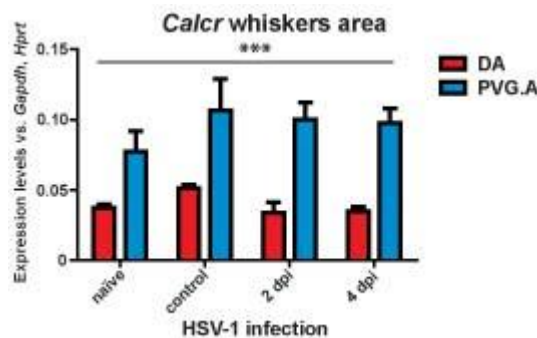
To find which of these three genes is most likely the candidate for regulating incidence of HSE we proceeded to the comparison of the mRNA expression of these three genes. We did not find any difference in the mRNA expression of the *Ccdc132* gene between the DA and the PVG rats. On the other hand in the mRNA expression of the *Tfpi2* gene there were significant differences between the two strains only in the trigeminal ganglia and the brain stem, but not in the whiskers' area. We therefore concluded that this might be a consequence of the infection rather than a cause, since in the whiskers' area there were no differences in the expression of *Tfpi2*.

7.1.7 Congenic lines covering *Hse1*



Breeding of congenic lines covering *Hse1* is ongoing. Congenic rats having an insert of PVG sequence on chromosome 4 on a DA background, covering *Hse1* have been infected. These congenic rats were all resistant to HSE development, further confirming the confidence interval. The PVG fragment on the DA background is still large; we aim to reduce by further breeding the size of the insert to *Calcr*.

7.1.8 Calcitonin receptor gene as a possible candidate for regulation of HSE in DA rats



The only gene, in which we found significant differences in the mRNA expression between DA and PVG rats in the whiskers' area, was the calcitonin receptor *Calcr* gene (Pondel 2000; Ernst, Morgenthaler et al. 2007; Hamza, Higgins et al. 2007; Shen, Crotti et al.

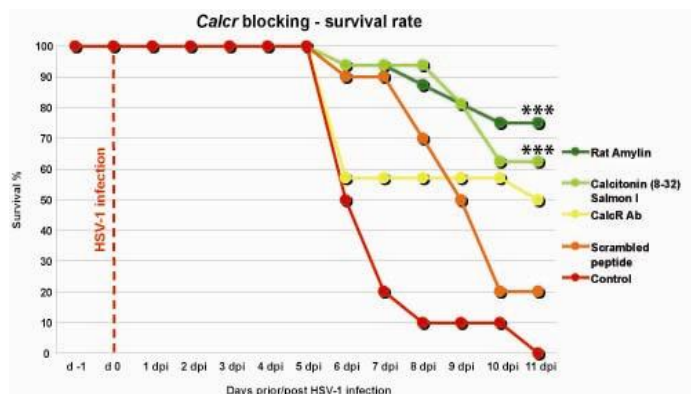
2007; Morfis, Tilakaratne et al. 2008; Naot and Cornish 2008). Interestingly, PVG rats had significantly higher expression of this gene both in naïve rats, vehicle-injected controls and HSV-1 infected rats at 2 and 4 dpi.

7.1.9 Functional studies

Once having a candidate gene for regulating HSE we turned to mechanistic studies to elucidate a possible disease regulatory effect of the calcitonin receptor.

Cell culture studies have shown that both fibroblasts and neurons isolated from fetuses and from adults of both DA and PVG strains were equally susceptible to HSV-1 infection. Neither treatment with rat Amylin, an agonist to the calcitonin receptor, nor treatment with CalcR antibodies influenced viral replication in the cells in culture. This

most likely indicates that the cross-talk of the cells among different cell-types in the living organism is of crucial importance for the protection against HSV-1 spread to the CNS.



We therefore attempted to manipulate the calcitonin receptor and to influence disease outcome *in vivo* in infected rats. Thus we injected into the whiskers' area of DA rats at the age of 44 days, *i.e.*

one day prior to HSV-1 infection, different substances modulating calcitonin receptor signaling. Remarkably, survival of the susceptible DA rats was significantly improved after pre-treatment with calcitonin receptor agonist Rat Amylin and calcitonin receptor antagonist Calcitonin (8-32) Salmon I (Hilton, Dowton et al. 2000) as compared to controls injected with vehicle or with a scrambled peptide, that is, the Amylin amino-acid sequence in reverse order.

7.1.10 Conclusions regarding HSE regulation in DA and PVG rats

We demonstrated in this study that the genomic region identified in an F2 cohort of intercross between the susceptible DA and the resistant PVG strain named *Hse1*, with *Calcr* as the main candidate gene, regulated susceptibility *vs.* resistance to HSE. Further studies will be needed to elucidate the role of CalcR in susceptibility to HSE in human studies and to put it in the focus of a therapeutic approach.

7.2 GENETIC STUDIES IN BN-SHR RECOMBINANT INBRED LINES



The resistant BN and the susceptible SHR rat

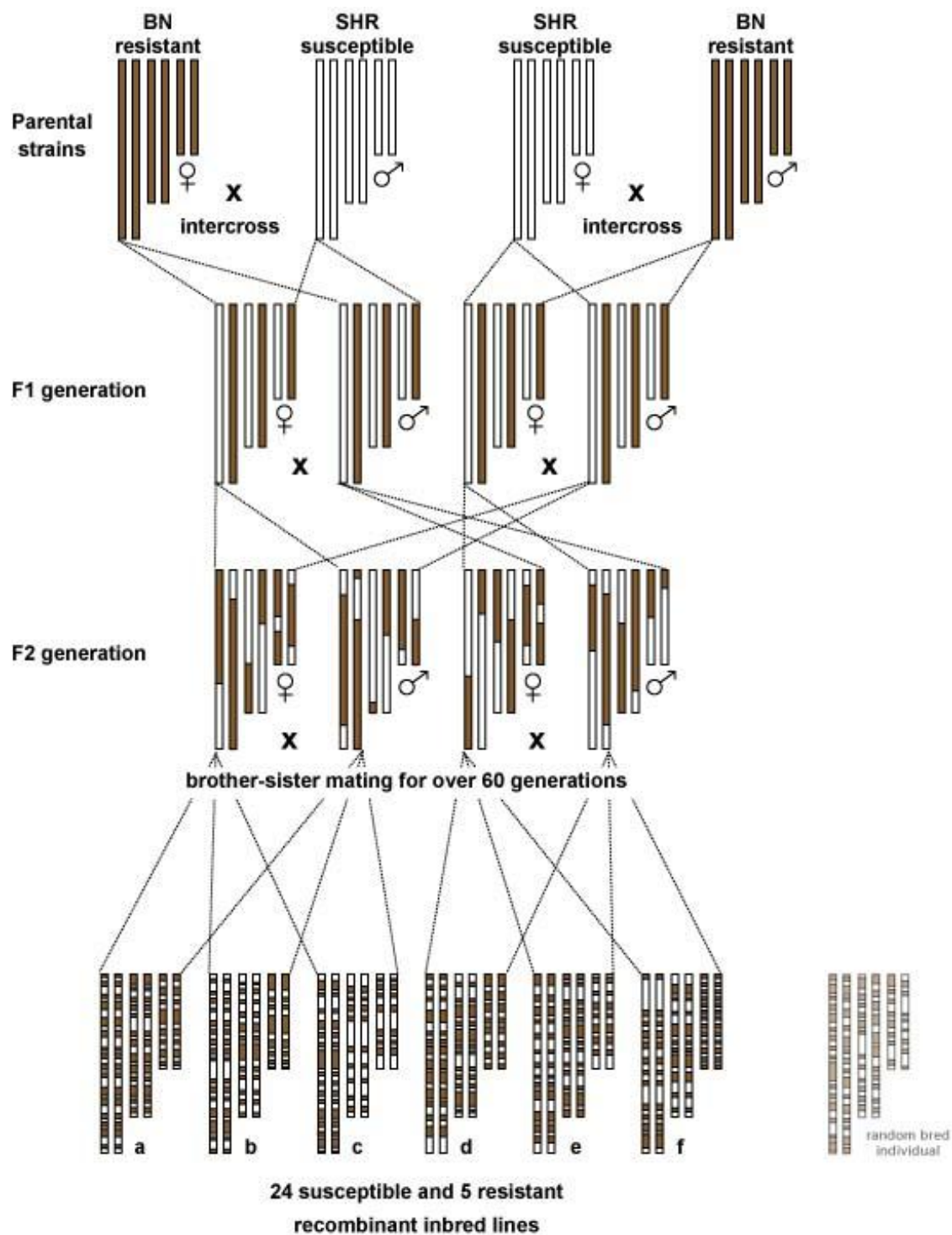
Studying susceptibility and resistance in different inbred rat strains we observed that BN rats were resistant to HSE in spite of the fact that they shared the DA genotype of the DA rats in the

Hse1 region.

In these experiments, another inbred rat strain, SHR, showed the same degree of susceptibility towards HSE as the DA rats. Our attention was drawn in an early stage upon a well characterized, SNP-genotyped panel of existing recombinant inbred rat lines existing in Prague and we have obtained rats from 29 of the existing 31 lines.

Recombinant inbred lines result from an intercross of two different inbred rat lines.

Individuals of the F2 generation are selected in pairs and their offspring is further bred by brother-sister mating for many generations, so that their genomes will contain a mixture of small fragments of the parental genomes, but homozygous for all the alleles, all individuals being genetically identical within the line, but different from the other line.



7.2.1 HSV-1 infected the CNS of the BN strain resistant to HSE

Starting from the experience observed in PVG rats, that HSV-1 did not penetrate either into the peri- or endoneurium of the peripheral nerves, nor into the trigeminal ganglia or the brain stem, we were surprised by the very interesting immunohistochemistry finding, that HSV-1 was widely spread not only into the epi-, peri- and endoneurium of the peripheral nerves, but also into the trigeminal ganglia and the CNS of the BN rats.

Interestingly, the fact that BN rats were identical to DA rats in the *Hse1* region corroborated this observation, however less immune cell infiltration could be observed in the brain stem of the BN than in the susceptible SHR rats.

7.2.2 Phenotyping of the recombinant inbred lines

We infected 45 day old rats of the 29 available inbred rat lines and observed them for disease incidence and development of the body weight curve. Five RILs did not present clinical symptoms of disease development, the rest got diseased at 3 – 5 dpi and the majority died at around 6 dpi.

7.2.3 Linkage analysis of the results

Genetic analysis of the phenotype results in the existing database (Pravenec, Klir et al. 1989; Mueller, Goel et al. 2006; Atanur, Birol et al. 2010) has identified a significant QTL, *Hse6* on chromosome 4 regulating disease incidence and weight loss between d0 and 10 dpi, not identical with *Hse1*, besides additional suggestive QTLs on chromosomes 1, 9 and 10 as *Hse7* and others.

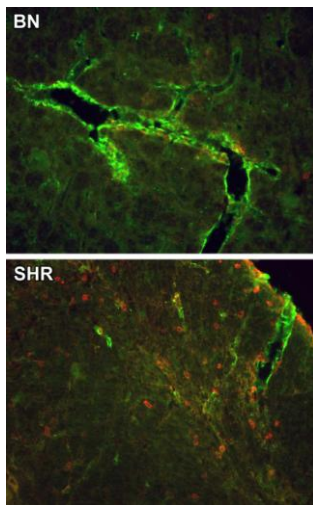
7.2.4 Identification of candidate genes

Hse6 consists of approximately 220 genes, many of which are associated with immune regulation, especially that of the natural killer and dendritic cell function. Sequence comparison between the BN and the SHR genomes show over 7,000 SNP polymorphisms, 750 short insertions or deletions and 55 large deletions, but only 24 genes can be identified with non-synonymous SNPs out of which only four are *cis*-regulated (*Leprecan-like 2*, *Vamp1*, *Vwf* and RDG1562378), however Von Willebrand factor homolog gene, *Vwf* was the only one with mutation at the essential splice-site and had 2 non-synonymous mutations. Moreover, *Vwf* was *cis*-regulated in 6 tissues including the brain, while the RDG1562378 gene was not *cis*-regulated in the brain.

7.2.5 Von Willebrand factor gene

After identifying *Vwf* as a candidate for regulation of susceptibility *vs.* resistance in the parental SHR and BN strains and in the recombinant inbred lines between these two

strains we attempted to visualize differences in the parental strains by immunohistochemistry.



Vwf and NK cell staining in the brain stem of BN and SHR

Vwf is a protein synthesized in the endothelial cells and it is stored in the intracellular granules. It is a mediator between the blood vessel wall and the platelet and functions as an anti-hemophilic factor carrier facilitating hemostasis (Pendü, Terraube et al. 2006). It has also been shown to have an important role in preserving the integrity of the blood-brain barrier.

In the whiskers' area and the brain stem of the resistant BN rats Vwf staining clearly delineated the wall of the blood vessels restricting to a great extent penetration of the NK cells into the tissue as compared to the SHR rats in which Vwf positivity was less structured and NK

cells were widely spread in the adjacent tissue (Kveberg, Jimenez-Royo et al. 2010).

7.2.6 Conclusions regarding HSE regulation in DA and PVG rats

Our study has identified HSV-1 spread to the CNS in both BN and SHR rats, however different pattern of NK cell penetration into the virus-affected compartments, supporting the hypothesis that differences in immune response to HSV-1 infection might play the major role in the development of HSE.

Linkage analysis of the phenotypes observed in 29 RILs established between these rat strains revealed a significant QTL associated with HSE pathogenesis, with the Von Willebrand factor as a possible candidate gene. Further studies will be needed to study the role of *Vwf* in susceptibility to HSE at a molecular level and in patients in order to reveal if it is a candidate for therapeutic studies.

8 CONCLUSIONS AND FUTURE PROSPECTS

8.1 PAPER I

Host strain-dependent difference in susceptibility in a rat model of *Herpes simplex* type-1 encephalitis.

The establishment of a model of susceptibility vs. one of total resistance to HSE in two inbred rat strains has confirmed the utmost importance of the use of genetically well characterised strains of rats in particular and of laboratory animals in general. By choosing a well-defined HSV-1 strain, an optimal virus-dose, a new inoculation site targeting the trigeminal nerve, an optimal age of the rat at infection and inbred rat strains the novel HSE model was created. The new dichotome model has opened possibilities to study many aspects of HSE as pathogenesis, the role of the immune system in the disease development and the genetic regulation of the phenotypes.

8.2 PAPER II

Influence of Perineurial Cells and Toll-Like Receptors 2 and 9 on *Herpes simplex* Type-1 entry to the Central Nervous System in a Rat Model of Encephalitis.

Compared to the resistant PVG rats low levels of virus detection by *Tlr2* and *Tlr9* at early time points after infection and slower recruitment of macrophages seem to be predisposing viral replication in the perineurial cell layer and its consecutive propagation to the CNS in the DA rats. The findings can serve as background to treatment studies. The different patterns of immune cell recruitment might be a consequence of the different mechanisms of HSV-1 spread in the periphery. The cause is more likely to be found in the genetically regulated differences in the properties of the cells in the perineurial layer within the two strains.

8.3 PAPER III

***Calcr* is a candidate for regulation of susceptibility to *Herpes simplex* type-1 encephalitis in the rat.**

Positioning *Calcr* as the main candidate gene of regulating HSE incidence in F2 offspring of susceptible DA and resistant PVG cross might be an indication of an existing function permitting or restricting HSV-1 penetration to the CNS. Additional studies will be necessary to explain the mechanism of how calcitonin receptor interferes with HSE-1 at the molecular level. Once the mechanism understood, *Calcr* might be target of therapeutic intervention. Since most humans harbor HSV-1 in their sensory ganglia after the primary infection in the early childhood, further investigations will be needed to reveal if *Calcr* has any role in the development of HSE in patients, in preserving the large HSV-seropositive population from HSE or in maintaining a small part of the population HSV-seronegative throughout lifetime.

8.4 PAPER IV

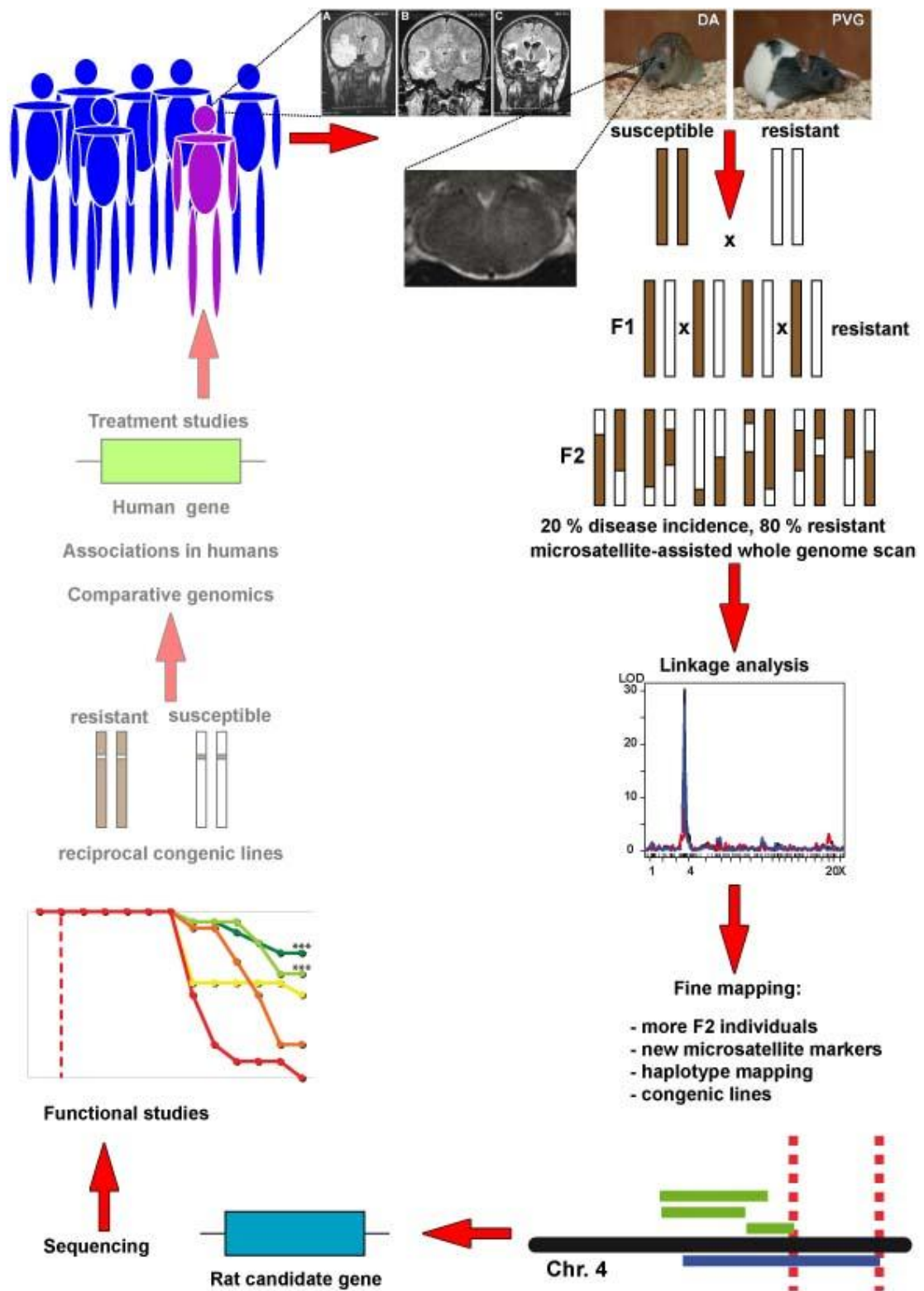
***Vwf* is the candidate gene for regulation of susceptibility to *Herpes simplex* type 1 encephalitis in rat recombinant inbred lines.**

Since HSV-1 penetrated after peripheral infection into the CNS of both susceptible SHR and resistant PVG rats, *Vwf* positioned as the candidate gene regulating susceptibility to HSE in these strains might also have a role in human HSE. Further studies will possibly reveal if an insufficiency in *Vwf* expression can explain why only a very small portion of the population harboring HSV-1 in their sensory ganglia develops HSE.

8.5 GENERAL CONCLUSIONS

The results found using our rat model have shown the importance of the early innate immune responses in preventing encephalitis after peripheral infection as well as the specific cell types which might have a role in restricting or facilitating HSV-1 entry to the neural compartment. Two known genes have been also found in two different rat populations which most likely influence susceptibility to vs. resistance against HSE. Further studies using samples from HSE patients compared to appropriate controls are needed to test the relevance of our findings for the human condition. Genotyping studies in humans with HSE have revealed deficiencies in the expression of the intracellular UNC-93 protein and TLR3, leading to impaired Interferon β responses. Most likely, there are several genes of key importance for HSE development, the dysfunction of which alone or together can predispose to HSE under certain conditions.

HSV-1 is a very complex ubiquitous virus and HSE is a rare disease. Therefore, in spite of huge efforts invested in their study throughout several decades, many questions for the pathogenesis of HSE have remained unanswered. It is very likely that neither the virus itself as in terms of latency, dose, neurovirulence etc. nor the host organism as in terms of genetics is alone responsible for the development of HSE, but both parts together contribute in variable proportions. A solution of the HSE puzzle will be the joint effort of scientists from the field of virology, immunology, genetics and others.



9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Herpes simplex virus, munsårsvirus infekterar i tidig ålder flertalet människor världen över och efter det finns viruset dolt kvar i nervknutar i organismen utan att orsaka några symptom. Olika faktorer som till exempel stress, mens eller sol kan göra att viruset aktiveras och bildar blåsor runt läpparna, så kallat munsår. Denna avhandling avser de sällsynta fall (ungefär 2-3 människor per million per år) där *Herpes simplex* virus orsakar en mycket svår hjärninflammation. Om sjukdomen upptäcks och kan diagnostiseras i tid, kan behandling med antivirala läkemedel, först och främst Aciclovir rädda livet hos de flesta patienterna, men neurologiska men från lindriga till mycket svåra förekommer ofta hos de överlevande.

Målet med denna avhandling var att skapa en ny råttmodell för hjärninflammationen orsakad av *Herpes simplex* virus, att fördjupa sig i de mekanismer som styr den tidiga immunreaktionen efter perifer infektion samt att försöka förstå om ärftliga egenskaper hos värdorganismen kan styra resistens eller känslighet.

Individer av inavlade råttstammar är lika som enäggstvillingar i sin arvs massa, men skiljer sig väsentligt från andra inavlade stammar. Vi har funnit en råttstam, DA, som insjuknar i dödlig hjärninflammation efter infektion med *Herpes simplex* virus injicerat ensidigt i morrhårsområdet. Samtidigt har vi funnit en annan stam, PVG, som förblev resistent även efter infektion med mångdubbel dos av virus. Vi har beskrivit denna dubbla modell av känslighet/resistens med en lång rad metoder som sträcker sig från olika molekylärbiologiska metoder till virusodling från vävnad och magnetkameraundersökningar.

DA och PVG råttstammarna har varit basen för uppföljningsstudier med vävnadsprover tagna 12 timmar och 1, 2, 3, 4 dygn efter virusinjektion. Vi undersökte särskilda molekyler som känner igen om viruset har kommit in i organismen och skickar vidare signaler till andra element av immunsystemet att agera och förgöra viruset och de redan angripna cellerna. I den känsliga stammen hittade vi mindre mängd av dessa molekyler i vävnadssnitten och som en följd skedde mobiliseringen av viktiga typer av immunceller också senare i dessa råttor. Utöver detta har vi kunnat konstatera att spridningen av viruset ägde rum på helt olika sätt i DA och PVG stammarna. Medan viruset i den känsliga stammen etablerade sig och infekterade cellerna som omgärdar och skyddar nerverna, kunde viruset i den resistenta stammen inte tränga sig in i närheten av dessa nerver. Resultaten pekar på att olika ärftliga egenskaper av cellerna runtom nerverna i de två råttstammarna skulle kunna bidra till sjukdomskänsligheten eller resistensen.

Parallellt med detta projekt undersökte vi med andra metoder tecken på ärftliga faktorer som kunde bidra till skillnaden mellan DA och PVG. Vi har korsat dessa två råttstammar och konstaterat att första generationen avkomma är totalt resistent, medan en fjärde - femtedel av andra generationen avkomma blir sjuk och resten förblir motståndskraftig. Vi har noterat vilka av dessa råttor som blev sjuka och vi har

analyserat vävnadsprover för att iaktta arvsmassan hos varje råtta. Individer i den andra generationen har slumpmässigt ärvt en del av sin arvsmassa från den ena, och resten från den andra stammen. Det finns markörsekvenser i arvsmassan som annars inte spelar någon roll för ärftligheten, men som skiljer sig storleksmässigt mellan de olika inavlade stammarna. Med hjälp av dessa sekvenser har vi kunnat identifiera vilka fragment av arvsmassan som ärvt från DA och vilka från PVG råttor hos de olika individerna av den andra generationen. Dessa data matades in i en specialskapad datormjukvara som har funnit vilken del av arvsmassan som möjligen styr sjukdomsmottagligheten. Denna del av arvsmassan innehåller en rad gener. För att exakt kunna finna vilken av dessa gener som kan orsaka *Herpes simplex* hjärninflammation (HSE) har vi jämfört våra data med en lång rad andra inavlade stammar och med hjälp av andra avancerade molekylärtekniska metoder kunde vi föreslå en gen, calcitonin receptor genen, som en möjlig kandidat för boven i dramat. Vi bekräftade sannolikheten av att calcitonin receptor genen styr sjukdomsmottagligheten genom att före infektionen behandla råttorna med olika substanser som påverkar receptorn. Dessa substanser har höjt överlevnaden till 75 % hos behandlade och infekterade råttor jämfört med 0 % hos icke-behandlade, infekterade råttor av den känsliga stammen DA. Hypotesen kommer att bekräftas ytterligare med hjälp av speciellt avlade DA råttor, som i sin arvsmassa bara kommer att ha calcitonin receptor genen från den resistenta PVG stammen. Ifall dessa råttor förblir skyddade från hjärninflammation, bekräftar detta calcitonin receptor genens roll i åkomsten.

Andra inavlade råttstammar kan också vara känsliga eller resistenta för *Herpes simplex* virus orsakad hjärninflammation. Vi har undersökt en annan population av korsning mellan den känsliga SHR och den resistenta BN stammen också, bestående av olika linjer som har inavlats under mer än 60 generationer. Arvsmassan av dessa linjer är redan väl dokumenterad vad gäller bidraget av de ursprungliga stammarna i deras arvsmassa. Genom att undersöka sjukdomsutvecklingen efter infektion i dessa linjer och analysera resultaten i en annan redan existerande datormjukvara kunde vi identifiera en klump av gener som möjligen har roll i regleringen av immunsystemet och känsligheten för hjärninflammationen. Vi har sedan uteslutit de flesta av dessa gener som inte kan vara bidragande och har slutligen identifierat en annan kandidatgen som också kan spela roll i ärftligheten av känslighet. Denna nya gen bildar ett protein, von Willebrand faktor homolog, som har en roll i att skydda hjärnans integritet mot penetrerande, skadliga immunceller.

Våra resultat ger oss en bild av vad som händer efter infektionen med *Herpes simplex* virus på molekylär- och vävnadsnivå och öppnar möjligheten att kunna studera om det hos människor är samma gener som hos råttor som kan leda till sjukdomsutveckling. Ifall dessa geners roll också kan bekräftas hos patienter med *Herpes simplex* virus orsakad hjärninflammation, kommer vi att ha en stark utgångspunkt för att med hjälp av modellen kunna utveckla bättre läkemedel och terapeutiska metoder för behandling av sjukdomen.

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In 1998, Oleg Gazenko, who worked on the Soviet space program during the Sputnik era, said, “The more time passes, the more I’m sorry about it... We did not learn enough from the mission to justify the death of a dog.” (Encyclopaedia Britannica online)



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* * *

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