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Genetic regulation of nerve injury-induced neurodegeneration and inflammation

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ABSTRACT

Neurodegeneration and inflammation in the central nervous system (CNS) are hallmarks of several neurological disorders, including multiple sclerosis (MS), Alzheimer's disease and Parkinson's disease. The susceptibility of an individual to these conditions is complex, i.e. influenced by both genetic and environmental factors. To study the genetic component of complex traits, experimental models are valuable tools to control for the impact of environment and to perform genetic mapping in large sample size intercrosses between inbred strains. The studies included in this thesis are based on the finding that inbred rat strains respond differently to nerve injury with regard both to degree of neurodegeneration and inflammatory responses, and aim at describing the phenotypic differences between strains in response to nerve injury in order to identify genetic regions regulating these parameters. The ultimate goal is to identify candidate genes of relevance to human disease.

We first performed a genome-wide linkage study of the responses to nerve injury by ventral root avulsion (VRA) in an F2 intercross between DA and PVG rat strains. This identified four loci regulating the degree of neurodegeneration (*Vra1, 2*), T cell infiltration (*Vra2, 3*) and major histocompatibility complex (MHC) class II expression (*Vra4*). From these results, we can conclude that the complex responses to nerve injury can be genetically dissected and are regulated by independent (*Vra1, 3, 4*), as well as linked or identical loci (*Vra2*). The *Vra4* locus displayed a very strong linkage to MHC class II expression by microglia after injury (logarithm of odds, LOD 27.4).

Next, to position a candidate gene, *Vra4* was fine-mapped by use of an advanced intercross line between DA and PVG^{av1} strains. By additional use of haplotype maps, sequencing and expression analysis of genes in the region, the MHC class II transactivator, *Mhc2ta* was identified as the candidate gene. A polymorphism in the corresponding human gene, *MHC2TA*, was found to mediate differential expression of MHC class II transcripts and was genetically associated to the susceptibility to the three inflammatory disorders MS, rheumatoid arthritis and myocardial infarction.

In the third study, congenic rats where the *Vra4* region harboring *Mhc2ta* had been transferred from PVG^{av1} to DA and vice versa, were studied with regard to MHC class II expression in the CNS and susceptibility to the MS model experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein. The expression of MHC class II was determined by the *Vra4* allele and was thus reversed in the congenics compared to their respective background strain. In addition, *Vra4* alleles from PVG^{av1} transferred to the susceptible DA background genome conferred significant protection from clinical manifestations of EAE.

The phenotypic differences between the DA and PVG were further studied by analyzing the global gene transcription levels with microarrays. This identified a common response to VRA at a transcriptional level as well as strain specific patterns with inflammatory genes prevailing in the DA rats. In addition, two genes differing in expression between the strains, *C1qb* and *Timp1* correlated to the degree of neurodegeneration in genetically heterogeneous animals.

In conclusion; neurodegeneration and inflammation in the CNS can be genetically dissected in rat strains displaying phenotypic differences in the response to nerve injury, and identified candidate genes can be of relevance to human disease.

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Discrete gene loci regulate neurodegeneration, lymphocyte infiltration, and major histocompatibility complex class II expression in the CNS.
The Journal of Neuroscience, October 29, 2003, 23(30):9817-9823

II. Maria Swanberg*, Olle Lidman*, Leonid Padyukov, Per Eriksson, Eva Åkesson, Maja Jagodic, Anna Lobell, Mohsen Khademi, Ola Börjesson, Cecilia M Lindgren, Pia Lundman, Anthony J Brookes, Juha Kere, Holger Luthman, Lars Alfredsson, Jan Hillert, Lars Klareskog, Anders Hamsten, Fredrik Piehl and Tomas Olsson.
***MHC2TA* is associated with differential MHC molecule expression and susceptibility to rheumatoid arthritis, multiple sclerosis and myocardial infarction.**
Nature Genetics, 2005, 37, 486 - 494
*These authors contributed equally to the work.

III. Karin Harnesk*, Maria Swanberg*, Johan Öckinger, Margarita Diez, Olle Lidman, Anna Lobell, Erik Wallström, Tomas Olsson, Fredrik Piehl.
***Vra4* congenic rats with allelic differences in the class II transactivator gene display altered susceptibility to experimental autoimmune encephalomyelitis.**
Submitted manuscript.
*These authors contributed equally to the work.

IV. Maria Swanberg, Kristina Duvefelt, Margarita Diez, Jan Hillert, Tomas Olsson, Fredrik Piehl and Olle Lidman.
Genetically determined susceptibility to neurodegeneration is associated with expression of inflammatory genes.
Neurobiology of Disease, October 24, 2006, (1):67-88.

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LIST OF ABBREVIATIONS

ACI	AxC9935 Irish
AD	Alzheimer's disease
Aif1	allograft inflammatory factor-1
AIL	advanced intercross line
ALS	amyotrophic lateral sclerosis
BC	backcross
BDNF	brain-derived neurotrophic factor
BN	brown Norway
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CI	confidence interval
cM	centiMorgan
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dark Agouti
DASH	dynamic allele-specific hybridization
EAE	experimental autoimmune encephalomyelitis
eQTL	expression QTL
Gapd	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HHV	human herpes virus
HLA	histocompatibility leukocyte antigen
IFA	incomplete Freund's adjuvant
IFNγ	interferon-γ
IL	interleukin
LEW	Lewis
lm	littermate controls
LOD	logarithm of odds
LPS	lipopolysaccharide
MAF	minor allele frequency
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
Mb	Megabase
MBP	myelin basic protein
MHC	major histocompatibility complex
MHC2TA	major histocompatibility complex class 2 transactivator
MI	myocardial infarction
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
MSLR	mean signal log ratio
NGF	nerve growth factor
OR	odds ratio
PBC	peripheral blood cell
PCA	principal component analysis

PCR	polymerase chain reaction
PD	Parkinson's disease
pQTL	physiological QTL
PRKCA	protein kinase c-alpha
PVG	Piebald Virol Glaxo
QTL	quantitative trait locus
RA	rheumatoid arthritis
RI	recombinant inbred
rMOG	recombinant rat myelin oligodendrocyte glycoprotein
RNO	rattus norvegicus chromosome
RT-PCR	reverse transcriptase polymerase chain reaction
SD	Sprague Dawley
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
SSLP	simple sequence length polymorphism
TGF	transforming growth factor
Timp	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
VRA	ventral root avulsion

1 GENETICS OF COMPLEX DISEASES

The unique genetic setup of an individual will determine a vast number of physiological characteristics, collectively termed traits. If determined by a single gene, the trait is monogenic, or simple. Most traits, like height and weight, are however influenced by several genes and also by the environment. Such traits are termed complex and will follow a normal distribution in a population. Falconer extended this view to discontinuous characters such as presence of disease (see (Falconer, 1996)). The liability to common diseases that depend on many different factors could thus also be viewed upon as a continuous trait with a normal distribution, ranging from low- to high-risk individuals. Most common diseases, e.g. autoimmune diseases, cancers and type-2 diabetes display this pattern and are thus termed complex diseases.

Complex diseases are defined as being multifactorial with both environmental and genetic components. An individual's genetic setup combined with environmental factors such as infections, diet, exposure to chemical compounds and purely stochastic events influences the susceptibility to a specific disease. The sum of all these factors determines if disease will occur or not. As a result of combining different genes and environmental factors in different individuals, a complex disease will be causally heterogeneous in patients with the same diagnose. To identify the genetic components of a complex disease, co-inheritance of chromosomal regions with disease is studied in families or at a population level. This will lead to an estimate of the increased risk mediated by alleles at specific genomic locations.

Genetic variations with effect on a phenotype could arise from single base insertions, deletions or substitutions (single nucleotide polymorphisms, SNPs), gene copy number variations or from larger insertions, deletions or translocations within or between chromosomes. If the frequency of a variant is >1% in the population, it is named a polymorphism, or allelic variant. The terms polymorphism and allele will be used for the genetic variants underlying traits studied in this thesis, since they are studied at a population level. The term candidate gene will be used for the genetic entity affected by the polymorphism, not necessarily a gene. Of note, the nature of discussed alleles or polymorphisms could be entire haplotypes, a SNP or larger genetic differences.

Assuming the presence of many contributing genetic factors, each with relatively low impact on disease susceptibility or severity, one could question the use of studying the genetics of complex diseases. However, the current lack of good therapies for many complex diseases much depend on the poor knowledge of their etiology, where genetics could contribute to increased knowledge at several levels: i) an allele with low impact on disease susceptibility at a population level may have great impact at an individual level, ii) the redundancy of genes contributing to the same phenotype indicates that they modulate, and could thus identify, common pathways or biological processes, iii) different genetic factors may reflect clinical sub phenotypes, in turn useful for diagnostic and therapeutic purposes, iv) knowing the genetic background for a specific disease could allow targeted therapy, or even preventive therapy in high-risk individuals.

1.1 THE THRESHOLD MODEL

Complex diseases are genetically heterogeneous. The most extensively characterized allelic variants with impact on complex disease are histocompatibility leukocyte antigen (HLA) alleles, and also other genes conferring small- to modest risk increments are thought to be common variants in the population (Lohmueller et al., 2003). More rare variants will probably be more difficult to identify at a population level, but may still be important for complex disease susceptibility (Duerr et al., 2006). Since risk alleles can interact with each other and with the environment, even an important genetic risk factor will not be carried by all affected patients. Instead, the specific risk for an individual is made up by a unique assortment of genetic and environmental risk factors. In addition, risk alleles may vary between populations, as exemplified by the HLA region in autoimmune disorders.

According to the threshold model, all individuals may carry risk factors for a specific disease, but only if the combination of risk factors reaches a specific threshold will they lead to disease development (Figure 1).

1.2 GENETIC APPROACHES

There are two main ways to identify genetic regions or alleles underlying a phenotype; linkage and association. In both of these, genetic markers with known genomic location are used. These markers can be solely tools to track allelic origin and determine genetic location, or be the actual functional variants. The first markers to be used were RFLPs coupled with enzymatic digestion. Later simple sequence length polymorphisms (SSLPs), or microsatellites, easily amplified by polymerase chain reaction (PCR) were used. With the development of fast and large-scale genotyping techniques, SNPs are now widely used as genetic markers.

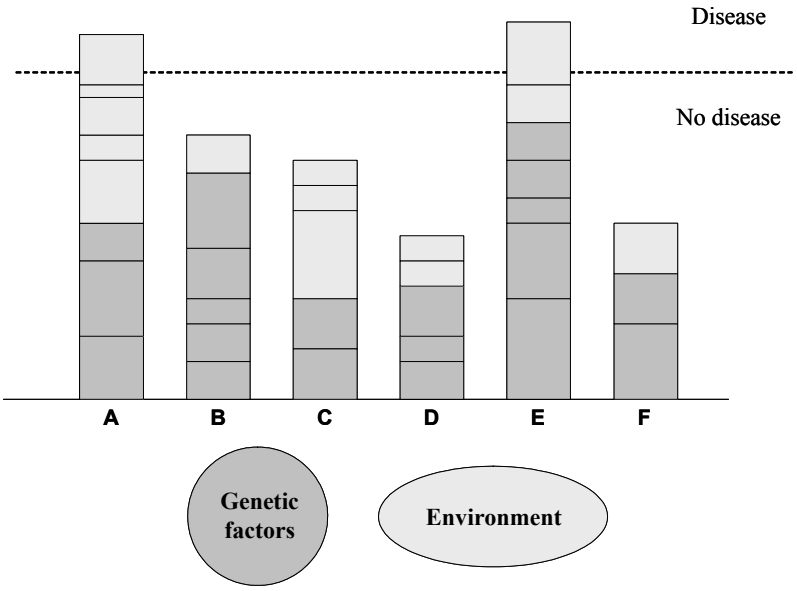

Figure 1. The threshold model. The combination of different genetic and environmental risk factors determines if an individual (A-F) will reach the threshold for disease development. The relative contribution from genetic and environmental factors varies, compare affected individuals A and E.

1.2.1 Linkage

Two loci on different chromosomes will be independently inherited by offspring due to random assortment of chromatids in parental gametes, according to the Hardy-Weinberg equilibrium. If the loci are instead located on the same chromatid, they can be in linkage disequilibrium as recombination is needed to separate them. Loci with a recombination frequency below 0.5 are thus linked. Genetic markers can be used to track a linked disease-associated polymorphism. Genetic linkage analysis is based on the co-segregation of genetic markers and disease in families. That is, if a genetic region contains risk alleles, it will more often be carried by affected family members. Linkage analysis to find disease associated genes thus relies on linkage at two levels: the linkage disequilibrium between a genetic marker and a genetic polymorphism, and linkage between the polymorphism and a phenotype such as disease.

A genetic region identified to be linked to a measurable phenotype is termed quantitative trait locus (QTL). The QTL describes the likelihood that one or more causative polymorphisms are present in the genetic interval, and its significance is presented by the base 10 logarithm of the likelihood ratio (logarithm of odds, LOD) score, i.e. the likelihood of individual genotypes given the presence of a QTL compared to the absence of a QTL. A LOD score of 3 thus indicates a 1000 times higher chance of having than not having a QTL in the studied population at that specific location.

Linkage analysis takes advantage of interval mapping, where the genotypes at surrounding markers are used to estimate the genotype at each position between them. This allows linkage to be used for whole genome scans and enable unbiased search of the entire genome for regions linked to disease, and not only point-wise analysis at genotyped marker loci. The first whole-genome scan by linkage analysis was conducted in tomato and was made possible by the full genome coverage of RFLP markers (Paterson et al., 1988). Linkage analysis in disease-affected families has been successful in identifying the genetics behind monogenic traits, but is also valid for mapping QTLs of complex disease. Identified QTLs will however be large since recombinations derive from only one or two generations resulting in poor marker segregation. The requirement of large family materials to obtain good power is a constraint, and most probably only QTLs with major impact will be identified. Linkage also excludes studies on non-familial forms of the disease. Two types of linkage analyses are performed in families, classical linkage and affected pair analysis. Classical linkage identifies recombinants and non-recombinants within large families and couples this information to presence of disease, while affected pair analysis search for genome regions shared by affected individuals more often than would be expected by chance.

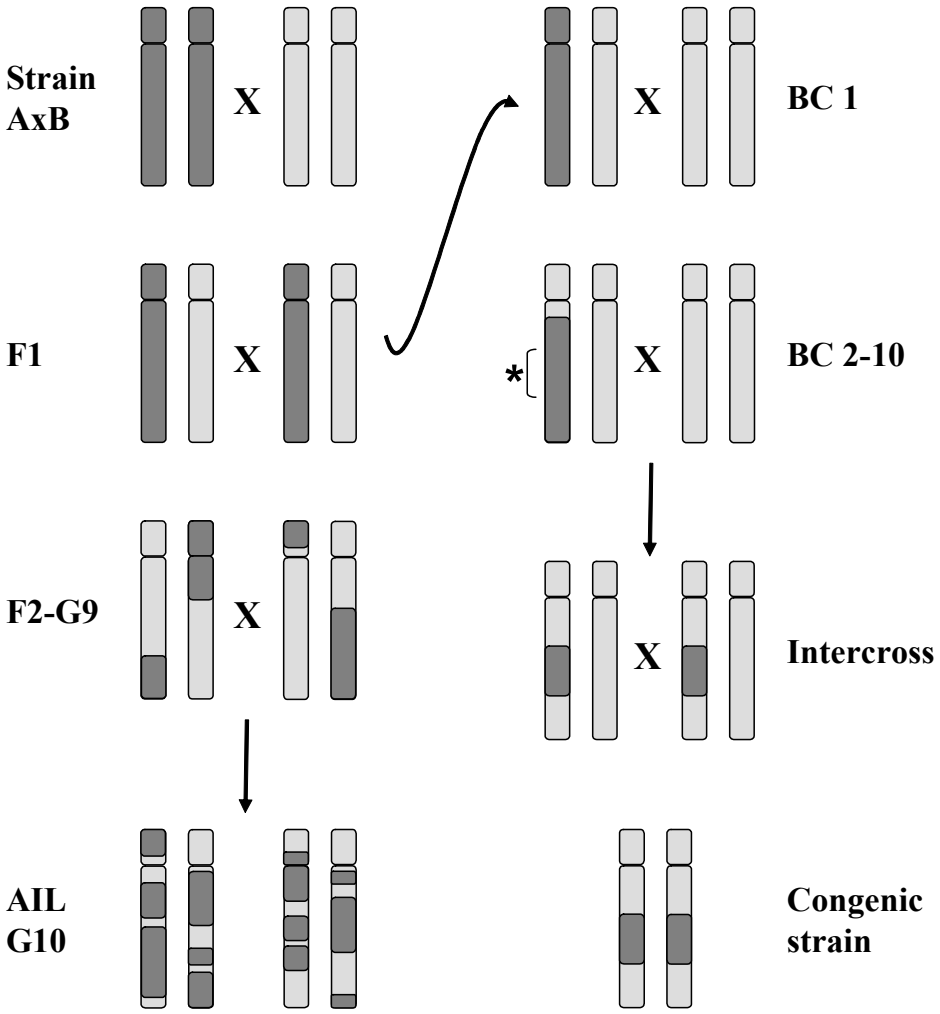

Figure 2. Breeding strategies for genetic mapping in experimental populations. For QTL mapping, an intercross between parental strains A and B will create an A/B heterozygous F1 generation. F1 intercross will produce a heterogenous F2 population that can be used for whole genome scans. To obtain a G10 AIL generation for QTL finemapping, fifty breeding pairs from the F2 to G9 generation are needed. For congenic breeding, an F1 individual is back-crossed to one of the parental strains. In each generation, an individual with the fragment of interest (*) is identified by genotyping and used for further back-crossing. By the 10^{th} generation, an intercross can produce homozygous congenics with theoretically <0.1% background contamination.

1.2.2 Association

Association analysis is performed at a population level and measures the frequency of alleles or haplotypes in affected compared to non-affected individuals. It is most often employed to test candidate genes or to narrow QTLs identified by linkage. Whole genome association studies have recently been made possible by the development of large-scale genotyping techniques such as microarrays. Since the included subjects are non-related, the resolution is high, and dense markers are needed to catch linkage disequilibrium between a marker and the causative polymorphism. Analysis of haplotypes, i.e. co-inherited polymorphisms, is a way to increase the power to detect association and can be useful when the causative polymorphism is not typed.

The significance level of an association is analyzed by different statistical methods depending on the mode of recessiveness or dominance and presented as p-values. The increased or decreased risk conferred by an allele or haplotype is presented as the odds ratio (OR). For complex diseases, a polymorphism conferring a modest increased risk could typically have an OR of 1.5.

1.3 ANIMAL MODELS

To study the mechanisms and genetics behind complex traits, animal models are invaluable tools. Throughout the years, inbreeding of mouse and rat strains have generated a panel of inbred strains that are homozygous across their genome, hence are identical within the strain, but unique compared to other strains. Based on the genetic setup, each strain displays strain-specific characteristics and phenotypes. To search for genetic elements regulating a specific trait, traditionally two or more strains differing with regard to the studied phenotype are selected and intercrossed. This will in the second generation produce heterogeneous offspring, all with unique assortment of the parental alleles across the genome (Figure 2). Linkage analysis can then be employed with a large sample size.

1.3.1 Inbred strains

An inbred strain is generated by inbreeding an isolated population for >20 generations. This will result in fixation of a certain allele and loss of all alternative alleles at each locus, creating homozygous, genetically identical individuals. Any recombination events occurring in the germline will thus be silent and not result in any new combination of alleles.

Inbreeding of mice and rats for laboratory purposes became systematic in the early 20th century and to date there are hundreds of inbred mouse and rat strains across the world. Mice became the first choice of geneticists while rats were used mainly to study physiology. The first genetic studies on rats were, however, performed already at the end of the 19th century on coat color. In this thesis, experimental work has been performed in the rat, which was the third mammalian genome to be sequenced, following man and mouse (Gibbs et al., 2004). In support for the relevance of mouse and rat genetics for human disease was the identification of an “ancestral core” across

the three species containing 95% of all annotated coding sequences and regulatory regions.

Inbred strains are the choice for experimental setups requiring replicate sampling, as any two animals matched by age, sex and environment can be used interchangeably. The specific nature of inbred strains includes the artificial state of homozygozity across the genome, allowing penetrance of recessive traits but loss of heterosis and imprinting effects.

1.3.2 Whole genome scans

Since offspring from intercrosses between inbred strains are of known descent, they can be treated as families and subjected to whole genome scans by linkage analysis. A whole genome scan is by definition unbiased. It aims at finding the genetic basis for a variable phenotype in a population by combining genetic and phenotypic information for each individual and performing analysis at a population level. This type of discovery-based research circumvents the need of, often limiting, pre-formed hypotheses. The studied phenotypes can be end-points like disease or no disease, or intermediate such as physiological parameters or expression levels of certain genes or proteins. These phenotypes may or may not be linked to the same QTL. In any case, a true QTL will contain the functional genetic variant underlying that phenotype and not trace secondary effects.

Crosses between inbred mouse and rat strains have so far identified more than 2000 QTLs, whereof almost 1000 are rat QTLs (Flint et al., 2005). It is likely that a single QTL contains several candidate genes. This could be due to closely linked genes with interacting or additive effects, or be a result of an experimental design with too low power to detect the lower impact of single-gene QTLs.

In order to perform genetic mapping by whole genome scans, a genetically heterogeneous population and polymorphic markers segregating between the parental origins are needed. Parental strains are often chosen based on their phenotypic differences, but phenotypically similar strains can also produce offspring displaying a variable phenotype. Whole genome scans are performed in backcross (BC) or intercross (F2) populations. BC has the advantage of allowing studies on epigenetic effects of imprinting but will lack homozygozity for all alleles originating from one of the parental strains, limiting interactions and excluding recessive effects from one founder strain. In contrast, an F2 population will display all three possible allelic combinations at each locus, thereby increasing complexity.

In both BC and F2 populations, informative recombinations are limited to one generation. In average, this results in one recombination per chromatid and limits the number of genetic markers needed for linkage analysis. A marker density between 10 and 20 centiMorgan (cM) is required for interval mapping in BC and F2. The rat genome database (RGD) has currently nearly 18500 markers in the SSLP database (www.rgd.mcw.edu). Still, the lack of polymorphic markers is problematic in QTL fine-mapping. The use of SNPs as markers and the development of haplotype maps are

possible solutions to this problem. Rat haplotype maps however may not be as informative as those for mouse depending on the more homogenous genetic background of inbred rat strains (Flint et al., 2005). As a measure of rat strain heterogeneity, 46% of 4338 studied SSLPs were polymorphic with between 2 and 13 different alleles in 48 inbred rat strains (Steen et al., 1999).

1.3.3 Advanced intercross lines

To narrow the relatively large QTLs identified in F2 and BC whole genome scans, the number of recombination events in the studied population must be increased. This can be done either by increasing the population size or, more practically, the number of intercross generations by use of an advanced intercross line (AIL). An AIL is created by repeated random intercrossing of at least 50 breeding pairs starting from the F2 generation from two inbred strains. QTL mapping can be performed on offspring from a selected generation, e.g. G10 (Figure 2). Analyzing animals in subsequent generations to G10 will, however, have only minor effects on reducing the confidence interval (CI), and will lead to fixation of alleles in the population. As the density of recombinations increases, the genetic map will be stretched out with each additional generation (t). The CI on the F2 scale will be reduced according to $[CI_2=CI_t/(t/2)]$. The 95% CI of a QTL with constant effect in populations of the same size will thus be reduced fivefold in the G10 compared to the F2 generation (Darvasi and Soller, 1995).

Due to the high density of recombinations, genetic mapping of an AIL requires dense genotyped markers. Without any practical constraints, high-resolution whole genome scans could be achieved with AILs, but for practical reasons, studies are focused on fine-mapping previously identified QTLs.

1.3.4 Congenic strains

A congenic strain is created by transferring a specific genomic fragment from a donor strain to a recipient strain genome (Figure 2). This strategy gives the possibility to study the biological effects of the introgressed alleles, i.e. the effects of a QTL can be isolated and dissected by functional studies. The random assortment of parental alleles across the genome in the intercross where the specific QTL was identified is however lost in the congenic strain. This will limit the effects of interactions between alleles of different parental origin. Interactions involving alleles in the congenic fragment will thus be limited to homozygous recipient loci.

By back-crossing for ten generations, the contaminating donor genome outside the selected fragment is theoretically less than 0.1%. An alternative is the speed congenic approach, where selection on the congenic fragment is coupled with assessing the background genome contamination by markers evenly dispersed across the genome. The background will be reduced by in average 50% in each generation, but due to random allelic combinations the reduction will follow a normal distribution in the offspring. By selecting founders in each generation with the least background, a 0.1% pure congenic can be obtained by five generations (Wakeland et al., 1997).

Congenic strains can be used to identify candidate genes, but are also useful for physiological and pharmacological studies without knowing the underlying candidate gene. In order to fine-map QTLs, overlapping recombinant congenic strains can be generated and compared to identify the minimal shared fragment in strains with retained phenotypic effect. This strategy is referred to as congenic mapping.

As a complement to congenic rat strains, transgenic rats were first developed in 1990 (Mullins et al., 1990). Lack of pluripotent rat embryonic stem cells has so far hindered the generation of stable knockouts. The first step towards rat knockouts was the successful cloning of rats by nuclear transfer (Zhou et al., 2003), opening the possibility for homologous recombination in somatic cells instead of embryonic stem cells. The recent use of RNA interference to disrupt gene function, either through short hairpin- or single-stranded RNA, has the advantage of its dominant nature, circumventing the need for cloning (Dann, 2007). Genes can also be selectively knocked out by coupling random mutagenesis induced by N-ethyl-N-nitrosurea, ENU, with screening for mutations in genes of interest and breeding onto a suitable genetic background (Smits et al., 2006).

2 NEURODEGENERATION AND INFLAMMATION

Neurodegeneration and inflammation are coupled phenomena in central nervous system (CNS) disorders of both neurodegenerative and inflammatory etiology. Primarily neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) are characterized by a local inflammatory response in the CNS dominated by activation of microglia (reviewed in (Wersinger and Sidhu, 2006; Rogers et al., 2007)). The presence of T cells in the CNS of AD patients has also been reported (Itagaki et al., 1988). The primarily inflammatory disease multiple sclerosis (MS) is characterized by demyelination and chronic inflammation, but also by axonal damage in lesions (Ferguson et al., 1997; Trapp et al., 1998) and the degree of neurodegeneration may in fact be the best corresponding parameter to clinical disability in MS (De Stefano et al., 1998). Thus, any of the two processes neurodegeneration and inflammation in the CNS will have implications for the other.

2.1 NEURODEGENERATIVE DISORDERS

Neurodegeneration is a feature of several neurological pathologies ranging from MS, stroke, polio and physical injury like head trauma to classical neurodegenerative disorders. Neurodegeneration can thus both be secondary or causative to disease. Primarily neurodegenerative disorders include AD, leading to dementia due to loss of cholinergic neurons, PD, characterized by loss of nigrostriatal dopaminergic neurons and amyotrophic lateral sclerosis (ALS), where motoneurons are lost. Patients may also display mixed clinical phenotypes.

The neurodegenerative disorders AD, PD and ALS all have familial, monogenic forms. These are however rare and the much more common sporadic forms are in fact complex genetic diseases. There is a possible genetic overlap between the familial and idiopathic forms of a disease. Taking PD as an example, less than 10% of the cases have a strict familial etiology (Payami and Zareparsi, 1998). One of the rare, familial PD mutations targets the alpha synuclein gene. This gene product was found to be associated with Lewy body formation in PD (Spillantini and Goedert, 2000) and common allelic variants of alpha synuclein to be associated with idiopathic PD (Kruger et al., 1999; Maraganore et al., 2006).

Since AD, PD and ALS are characterized by degeneration of distinct neuronal populations, factors affecting inherent vulnerability are probably involved in disease susceptibility. Such factors include nerve cell vulnerability, glial function and the local microenvironment. To date, existing treatment options are mainly symptomatic. As a complement to compensate for the loss of cell function or transmitter release, efficient therapy for these diseases should aim at slowing or stopping the degenerative processes. To do so, knowledge of the target cell susceptibility and the local cellular interactions is needed.

2.2 MULTIPLE SCLEROSIS

MS is a chronic, inflammatory disease with complex genetics. It is characterized by demyelination, inflammation, axonal damage and nerve cell loss. Patients are diagnosed after fulfilling certain criteria such as that lesions must be separated in both location and time (Poser et al., 1983; McDonald et al., 2001). The disease course may be primary progressive, secondary progressive or relapsing-remitting. Devic's disease, or neuromyelitis optica, is a severe form of demyelinating disease characterized by lesions restricted to the optic nerve and spinal cord and affects predominantly Asian patients. The inflammatory characteristics of traditional MS include cellular infiltrates in brain lesions and oligoclonal banding of proteins in the cerebrospinal fluid (CSF) that are caused by antibodies generated as a consequence of clonal expansion of B cells. Axonal damage is also evident and neurodegeneration is in fact a good correlate for functional loss (Ferguson et al., 1997; Trapp et al., 1998). Autoimmunity in MS is supported by the consistent genetic association to certain HLA alleles (DR15 and DQ6) (Jersild et al., 1973; Hillert, 1994; Godde et al., 2005), cellular infiltrates in the lesions, CSF oligoclonal bands and by animal models. In addition, the beneficial effects of immunosuppressive therapy strengthen the autoimmunity theory.

The etiology of MS is not known but has both genetic and environmental components. To add further to its complexity, the existence of clinical subtypes of MS suggests it to be a syndrome grouping related diseases that may well have different etiologies. If so, any study aiming at finding the genetic or environmental risk factors will increase power if able to discriminate between the different subtypes, something that has proven to be difficult in clinical practice.

The prevalence of MS follows a geographic pattern of areas with high (>30/100 000), medium and low (<5/100 000) prevalence (Kurtzke, 1993). High prevalence areas include northern Europe, the northern USA and Canada, southern Australia and New Zealand, medium prevalence areas include southern Europe, southern USA and northern Australia, and low prevalence areas include Asia and South America. In addition, high- and low-prevalence populations exist within these areas. While twice as many women compared to men develop the disease, men tend to have a more severe disease course. Adoptees do not acquire a familial increased risk for disease, but migrating from a low- to high risk area before adolescence increases the risk and vice versa (reviewed in (Kantarci and Wingerchuk, 2006; Giovannoni and Ebers, 2007)). In families with MS, the increased risk compared to the general population ranges from 300-fold for monozygotic twins to around 30-fold for first-degree relatives (Sadovnick et al., 1996). The concordance rate of MS is around 25% in monozygotic twins compared to 3% in dizygotic twins. This clearly demonstrates the polygenetic nature of MS, but also the impact of environment and stochastic events, since risk can, in part, be acquired and most individuals having a monozygotic twin with MS will not develop the disease. Environmental factors suggested to have impact on the risk for MS include smoking, vitamin D, infections such as human herpes virus (HHV) -6, Epstein-Barr virus (EBV), human endogenous retrovirus (HERV) and Chlamydia pneumoniae (Giovannoni and Ebers, 2007).

As mentioned above, the HLA region is the most consistently reproduced gene region associated to MS. Replicated associations are limited to a few genes; protein kinase c-alpha (*PRKCA*), MHC class II transactivator (*MHC2TA)* and interleukin (IL)-7 receptor (*IL7R*).

A region on human chromosome 17q was first found to be linked to MS (Sawcer et al., 1996) and the corresponding rat region was found to be linked to experimental autoimmune encephalomyelitis (EAE) (Dahlman et al., 1999b; Jagodic et al., 2001). The candidate gene in the region, *PRKCA*, was later found to be associated to MS in the UK (Barton et al., 2004) and two different allelic variants of *PRKCA* were found to be associated to MS in Finnish and Canadian populations, respectively (Saarela et al., 2006). Interestingly, the risk haplotypes showed a correlation with the transcription levels of the gene. Animal studies were also used as tools when identifying *MHC2TA* as candidate gene for MS. In Paper II, we reported association to MS, RA and MI, but the association to MS was weaker than for the two other diseases, and there was conflicting results from different control groups. Association of MS to *MHC2TA* has been confirmed in Spain (Martinez et al., 2007b) and the UK (Mihalova et al., 2007), but negative findings are also reported (see General discussion, Table 4). The most robust association to MS has been reported for the *IL7R*, first identified as a candidate gene in mouse EAE (Sundvall et al., 1995). Its genomic location at 5p14-12 was also pointed out in human linkage studies (Ebers et al., 1996; Oturai et al., 1999). Positive association to MS was reported from three smaller studies (Teutsch et al., 2003; Booth et al., 2005; Zhang et al., 2005), and confirmed in large materials (Gregory et al., 2007; Hafler et al., 2007; Lundmark et al., 2007). Genetically, the likely causal SNP (rs6897932) is located within the alternatively spliced exon 6 and this may have a functional effect on gene expression by disrupting a splicing silencer, thereby influencing the amount of soluble and membrane-bound isoforms of the protein (Gregory et al., 2007).

Of note, these three candidate genes were all first indicated in experimental animal models, confirmed in human materials and have allele-dependent gene expression.

2.3 ANIMAL MODELS

Animal models are widely used to study the pathology and etiology of human disease. The main advantages are the access of unlimited number of study subjects, the possibility of reducing complexity caused by environment and/or genetics, and ethical issues. An experimental animal model may aim at mimicking human disease, or be limited to specific sub phenotypes.

2.3.1 Nerve injury

Models for nerve injury have been extensively studied in the field of neuroscience with regard to injury responses, functional loss and regeneration. Compared to proximal injury, distal peripheral nerve transection is characterized by a higher degree of regeneration and increased number of surviving motoneurons. Ventral root avulsion (VRA) is a very proximal nerve injury in the rat where lumbar ventral roots containing

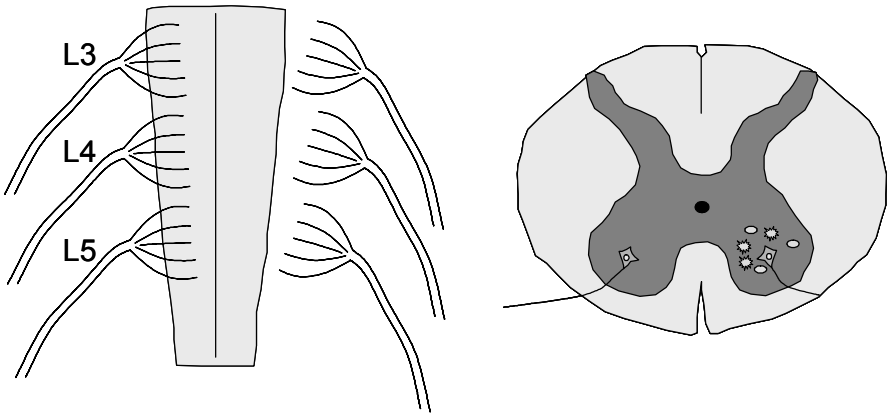

Figure 3. Schematic illustration of the VRA model. Ventral lumbar roots L3-L5 are unilaterally avulsed. A cross-section of the cord show axotomized motoneuron surrounded by activated glial cells.

axons from motoneurons residing in the ventral horn of the spinal cord are detached at the border between the central and peripheral nervous system (Koliatsos et al., 1994) (Figure 3). The resulting axotomized motoneurons and the surrounding glial cells will display classic features of a retrograde response involving chromatolysis, i.e. swelling of the cell soma, shifting of the nucleus and dispersal of Nissl bodies (Lieberman, 1971; Kreutzberg, 1982; Aldskogius and Svensson, 1993; Aldskogius and Kozlova, 1998). The underlying signals for these drastic changes have long attracted interest but still are mainly unknown (Cragg, 1970). The first message that injury occurred is thought to be mediated by influx of ions at the injury site resulting in retrogradely acting electrophysiological responses (reviewed in (Hanz and Fainzilber, 2006)). The motoneuron will in turn mediate as yet unidentified signals to surrounding glial cells that injury occurred.

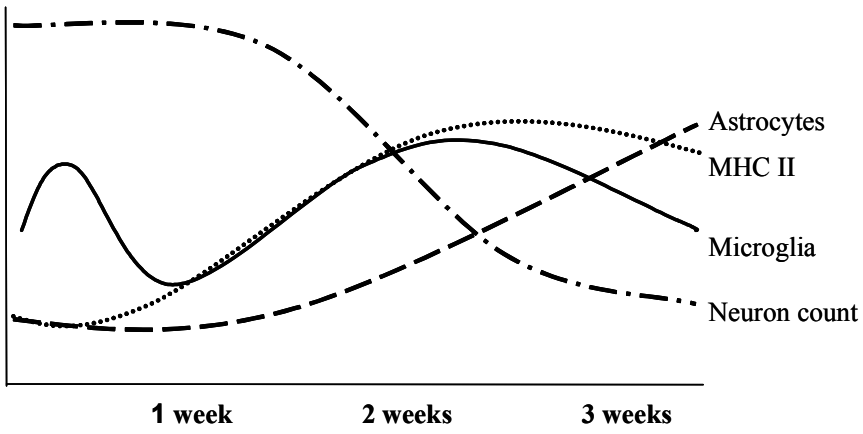

Figure 4. Kinetic pattern of the VRA response. Microglia display two phases of activation, the latter coinciding with decreased motoneuron counts. Adapted from Piehl and Lidman, 2001.

The glial response to VRA can first be observed one day after injury and follows a kinetic pattern of early morphological and metabolic signs of microglial activation such as up regulation of allograft inflammatory factor-1 (Aif1), followed by a later phase characterized by microglial major histocompatibility complex (MHC) class II expression and astrocyte activation. Loss of motoneurons will start around 1 week after VRA and reaches a steady state after around 3 weeks (Figure 4).

Axotomy-induced changes results in neurodegeneration of a varying proportion of motoneurons, depending on e.g. the proximity of the lesion to the cell body and the age of the animal. In adult animals, the resulting neurodegeneration displays both apoptotic and necrotic characteristics (Li et al., 1998). Signs of apoptosis include the delayed timing of cell death and structural changes. Accumulation of active mitochondria within the perikaryon and oxidative damage to nucleic acids and proteins may be contributing mechanisms (Martin et al., 1999). Axotomy in the adult rat also leads to increased expression of the apoptosis-related products Bax, Bcl-2, Bcl-X, and c-Jun in sensory and motor neurons (Gillardon et al., 1996; Baba et al., 1999). A functional role for anti-apoptotic factors is supported by the finding that delivery of a viral vector encoding anti-apoptotic Bcl-2 one week prior to VRA increased the survival of motoneurons by 50% (Yamada et al., 2001). In addition, rescue and increased choline acetyltransferase expression in surviving motoneurons could be mediated by delivering Bcl-2 together with glial cell line-derived neurotrophic factor (GDNF) after VRA (Natsume et al., 2002).

Although the responses to VRA follow a characteristic pattern (Figure 4), the intensity of the glial responses and the extent of motoneuron loss vary depending on the genetic background (Figure 5). By studying the responses to VRA in different inbred rat strains, the genetic effect on neurodegeneration as well as glial activation, including

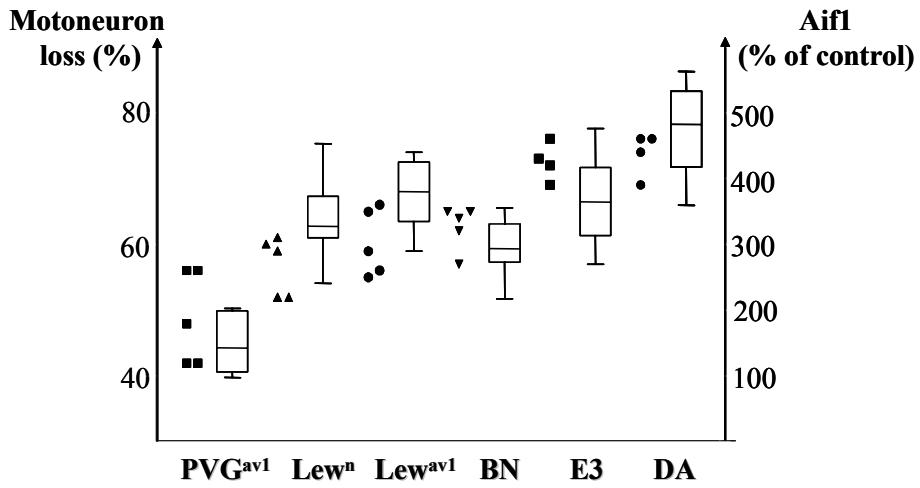

Figure 5. Strain differences in the VRA response. Motoneuron loss at 3 weeks and early microglial activation vary between and correlate within strains. Adapted from Piehl and Lidman, 2001

MHC class II up regulation, could mainly be attributed non-MHC genes (Piehl et al., 1999; Lundberg et al., 2001). There is a correlation between a more intense early glial activation and increased subsequent neuronal loss (Figure 5), arguing for a functional relation between these responses. However, the different parameters for glial activation show independent regulation, as exemplified by a very sparse MHC class II expression in the E3 rat (Lundberg et al., 2001). Among the studied rat strains, dark Agouti (DA) and Piebald Virol Glaxo (PVG) were identified as the most "susceptible" and "resistant" strains to VRA-induced neurodegeneration and inflammation. The DA rats thus display profound loss of motoneurons (80% compared to 60% in PVG) and a more pronounced glial activation as compared to the PVG rats.

2.3.2 Experimental autoimmune encephalomyelitis

EAE is an animal model for MS and can be induced in rodents by immunization with myelin components in adjuvant, or by transfer of encephalitogenic T cells. The resulting disease course, incidence, severity and histopathology will depend on the induction protocol, environment, age, size, sex and genetic background of the animal (reviewed in (Gold et al., 2006)). There is yet no spontaneous EAE model, but double-transgenic mice with myelin oligodendrocyte glycoprotein (MOG)-specific T cell receptors and MOG-specific IgH antibodies develop a severe form of EAE similar to human Devic's disease (Bettelli et al., 2006; Krishnamoorthy et al., 2006).

The disease course in EAE is determined by daily scoring of the animals, assigning higher scores as more severe symptoms develop. The scoring data is combined with measure of weight loss during the experiment, a phenotype that precedes clinical signs of EAE and can also indicate sub clinical disease (Figure 6). Most EAE models are characterized by ascending inflammation in the spinal cord, but immunization with recombinant rat MOG (rMOG) amino acid 1-125 from the N-terminus in incomplete Freund's adjuvant (IFA) results in distributed, focal lesions more resembling human MS (Storch et al., 1998; Weissert et al., 1998). The rMOG-induced EAE was therefore the model of choice in the EAE studies included in this thesis (Paper III).

As is the case for neuronal susceptibility to nerve injury, inbred rat strains display a varying degree of susceptibility to EAE. Interestingly, there is a correlation between the two phenotypes. The DA and brown Norway (BN) strains are EAE susceptible and lose relatively more motoneurons after VRA compared to the PVG and AxC9935 Irish (ACI) strains, which are EAE resistant. Like in human MS, the main determinant for susceptibility to EAE is the MHC haplotype (Williams and Moore, 1973; Gasser et al., 1975; Gunther et al., 1978). The MHC is also linked to severity of established EAE (Moore et al., 1980). As an example of the impact from the MHC, rats congenic for the MHC on the Lewis (LEW) background are resistant to rMOG-induced EAE if carrying the $RT1^{l}$ or $RT1^{w}$ haplotypes and susceptible if carrying the $RT1^{a}$, $RT1^{avl}$ or $RT1^{n}$ haplotypes (Weissert et al., 1998). Non-MHC genes also have an impact on susceptibility, as exemplified by studies on the PVG, LEW, ACI and DA strains, either congenic for, or naturally carrying, the $RT1^{avl}$ MHC haplotype. The non-MHC genes in these strains make the ACI and PVG strains resistant but the LEW and DA strains susceptible to rMOG-induced EAE (Weissert et al., 1998).

Whole-genome linkage analyses have been performed on five different rat intercrosses (Dahlman et al., 1999a; Dahlman et al., 1999b; Roth et al., 1999; Bergsteinsdottir et al., 2000; Becanovic et al., 2003). A meta-analysis performed on three of these intercrosses confirmed shared QTLs in different strain combinations and resulted in narrowed CIs (Jagodic and Olsson, 2006). Many shared QTLs in the combined analysis were not detected in each individual F2 cross, probably due to a lack of power caused by too few animals included and insufficient marker density. One example is *Eae19* on rat chromosome 15 (RNO15) that was previously shown to mediate protective effects in a DA.ACI congenic strain and in an independent DAxPVG^{av1} intercross (Sheng et al., 2005). This illustrates the complex nature of EAE genetics, with a redundancy of QTLs whereof many may show incomplete penetrance and highlight the need of combining and carefully evaluate experimental data.

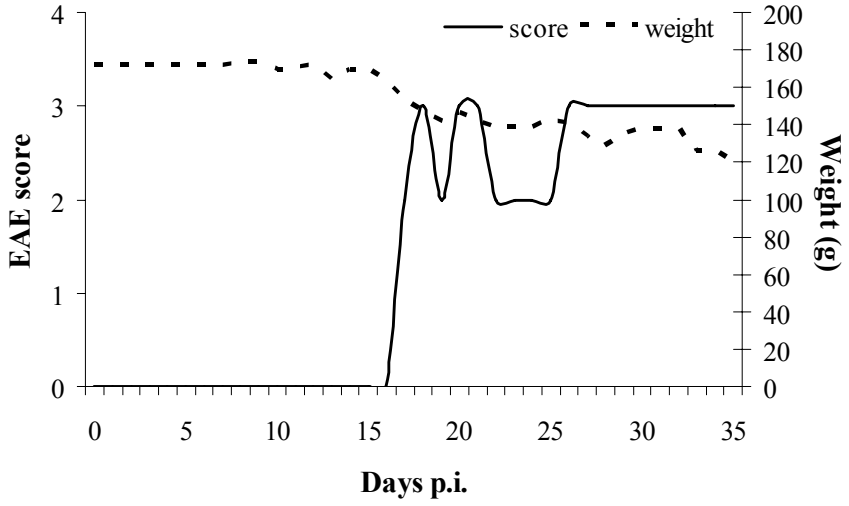

Figure 6. Example of EAE clinical score and weight in a rat with rMOG-induced EAE. The weight loss preceeds the onset of clinical score. EAE scores: 1: Limp tail, 2: Hind leg paraparesis, 3: Hind leg paralysis, 4: Tetraplegia, 5: Moribound or dead.

2.4 NEUROIMMUNOLOGICAL INTERACTIONS

The peripheral immune system and the CNS interact at several different levels. At a cellular level, immune cells patrol the CNS under normal conditions and accumulate during disease. At a molecular level, soluble factors mediate cross-talk between the two systems. The main communication pathways of peripheral and central cytokines are primary afferent nerves, directly through the BBB or through cytokines in the circulation that act on microglia via the circumventricular organs (Laflamme and Rivest, 1999).

The normal interplay between the immune system and the CNS during systemic inflammation contributes to sickness behavior responses through induction of pro-inflammatory mediators such as IL-1β, IL-6, tumor necrosis factor (TNF) and prostaglandins produced in the CNS (Hart, 1988; Dantzer, 2004). Systemic inflammation also has implications for chronic inflammatory and neurodegenerative diseases. Upper respiratory infections can be associated in time with relapses in MS, and systemic infections can also cause worsened symptoms in other chronic inflammatory diseases like RA and asthma. In addition, systemic inflammation and infection have implications for primarily neurodegenerative diseases like AD (Holmes et al., 2003). A reason could be priming of glial cells due to the neurodegenerative processes and hence a stronger subsequent response to systemic inflammation. In animal neurodegenerative models, microglia express low baseline levels of cytokines, but are primed to mount strong inflammatory responses upon further stimulation. Lipopolysaccharide (LPS) administrated to mimic systemic infection in mouse prion disease led to increased production of IL-1β and exaggerated sickness behavior (Combrinck et al., 2002). In addition, the systemic inflammation increased neuronal apoptosis (Cunningham et al., 2005).

There is thus a constant interplay between the nervous and the immune systems both during health and disease.

2.4.1 Glial activation

The glial cells of the CNS include oligodendrocytes, astrocytes and microglia. Oligodendrocytic processes wrap around axons, isolating them with their myelin sheaths and are targets for autoimmune attack in the process of MS. The astrocytes and microglia physically support neurons and maintain homeostasis in the normal brain but react rapidly upon any disruption of the local microenvironment.

In contrast to the ectodermal origin of other CNS cells, the microglia are derived from the mesodermal lineage. In the healthy brain, the microglia have a branched morphology and express low levels of MHC class I, II, cluster of differentiation (CD) 45 and beta-2 integrins such as CD11a, b and c (Akiyama and McGeer, 1990). Although apparently resting, the microglia are constantly active in surveilling the local CNS environment (Nimmerjahn et al., 2005). Upon disruption of homeostasis by disease, injury or stimulation by inflammatory mediators, the microglial morphology changes to a rounded shape, and they proliferate, become motile and upregulate expression of Aif1 (Tanaka et al., 1998) and surface molecules such as the complement

component 3 receptor CD11b (Graeber et al., 1988), MHC class I and II (Streit et al., 1989; Tooyama et al., 1990; Vass and Lassmann, 1990), CD45 (Masliah et al., 1991) and CD86 (B7-2) (Satoh et al., 1995). Activated microglia produce a number of inflammatory mediators such as complement, cytokines, reactive oxygen species and metalloproteinases. The arsenal of secreted products depends on the specific stimulus and includes both pro-inflammatory cytokines such as IL-1β, IL-6 and TNF (Giulian et al., 1986; Sawada et al., 1989; Peyrin et al., 1999) and anti-inflammatory cytokines such as transforming growth factor (TGF) β1 (Cunningham et al., 2002).

Astrocytes react upon injury to the CNS by a process referred to as reactive astrogliosis, or glial scarring. The astrocytes migrate to the site of injury, proliferate, upregulate intermediate filaments such as glial fibrillary acidic protein (GFAP), and form a scar surrounding the damaged region (reviewed in (Ridet et al., 1997)). This process is an attempt to restore homeostasis after injury, but will also limit the regenerative capacity of damaged neurons and axons. In addition, astrocytes react to microglial activation by responding to mediators like IL-1β and IL-6 (Giulian et al., 1994; Klein et al., 1997) and have active roles in the CNS interplay with the immune system. Expression analysis of interferon-γ (IFNγ) stimulated mouse astrocytes by microarrays demonstrated their capacity of transcribing a vast number of genes involved in both innate and adaptive immune responses including MHC class I and II, CD74, IL-18 binding protein and chemokine ligands 5, 9, 10 and 11 (Halonen et al., 2006).

Glial activation has both toxic and protective effect on neurons. Supernatants from *in vitro* stimulated microglia are toxic to neurons (Suzumura et al., 2006), but both astrocytes and microglia can produce TGFβ, which have neuroprotective properties (Flanders et al., 1998). Astrocytes can also produce neurotrophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and GDNF, and this production is increased by LPS and synergistically by IL1β and TNFα (Suzumura et al., 2006). The effects of glial activation will depend on the biological context and differ between acute and chronic states. As an example, the microglial response to inflammatory stimuli was shown to be altered during chronic inflammation *in vitro* (Ajmone-Cat et al., 2003) and *in vivo* (Ferrari et al., 2004). This may be due to similar events as in macrophages requiring an unresponsive and anti-inflammatory phenotype. Also microglial cells in culture acquired anti-inflammatory properties (secretion of NGF, TGFβ, prostaglandin E2 and IL-10) upon addition of apoptotic neural cells (De Simone et al., 2003).

Complement components have complex effects in the CNS and are produced locally by astrocytes (Rus et al., 1992), microglia (Graeber et al., 1988; Gasque et al., 1995), oligodendrocytes (Hosokawa et al., 2003) and neurons (Terai et al., 1997). The classical complement pathway can be activated by oligodendrocytes and myelin with or without myelin-reactive antibodies (Vanguri et al., 1982; Reindl et al., 1999). The high susceptibility of neurons and oligodendrocytes to the lytic effects of complement may be due to their low expression of complement inhibitors (Wing et al., 1992; Singhrao et al., 1999b). Expression of complement has been shown in MS (Sanders et al., 1986) and the neurodegenerative disorders AD (Eikelenboom and Stam, 1982), ALS (Kawamata et al., 1992), PD (Yamada et al., 1992) and HD (Singhrao et al., 1999a). Even though activated complement components contribute to the inflammatory state of

the diseased CNS, neuroprotective effects have also been reported. Inhibition of C3 in a mouse model of AD lead to increased amyloid deposits and neurodegeneration (Wyss-Coray et al., 2002) and sublytic concentrations of C5b-9 complexes are able to rescue oligodendrocytes from apoptosis *in vitro* (Rus et al., 1996).

2.4.2 Antigen presentation in the CNS

As the CNS was previously considered more or less isolated from the immune system under normal conditions, it was thought to lack functional antigen presenting capacity. It is now clear, however, that immune surveillance of the CNS occurs, that CNS antigens reach the circulation and that circulating lymphocytes traffic the normal CNS (reviewed in (Cserr and Knopf, 1992)). When activated T lymphocytes are transferred to the rat circulation they rapidly appear in the CNS tissue, where they reach a peak after between 9 and 12 hours, and exit within 1 to 2 days. If the T lymphocytes encounter the proper antigen bound to MHC class I or II molecules, they can remain in the tissue and initiate inflammation (Hickey et al., 1991).

MHC class I can be expressed on neurons, astrocytes, oligodendrocytes and microglia after exposure to IFNγ *in vitro* (Wong et al., 1984). The expression of MHC molecules *in vivo* was long considered to be restricted to non neuronal cells (Lampson, 1995), but this view has been revised. Thus, neurons have been shown to express MHC class I in experimental rat models (Olsson et al., 1989; Corriveau et al., 1998; Linda et al., 1998), and expression of MHC class I was shown to play a role for synaptic connections during development (Huh et al., 2000). A functional role for neuronal and glial MHC class I expression in adult animals is supported by findings that lack of MHC class I significantly increased synaptic stripping and impaired the regenerative capacity after nerve injury (Oliveira et al., 2004). In addition, rat neurons have been shown to constitutively express nonclassical MHC class I (*RT1-U*) and beta-2-microglobulin transcripts while classical MHC class I was predominantly expressed by glial cells and upregulated in response to nerve injury (Lidman et al., 1999).

The classical MHC class II expressing cells in the CNS are the microglia, which up regulate these molecules as part of their activation. Perivascular macrophages are another source of MHC class II, and during active inflammation, blood-derived antigen presenting cells can populate the CNS. MHC class II can also be expressed by a subset of astrocytes after exposure to IFNγ *in vitro* (Wong et al., 1984). The induction of MHC class II in astrocytes by IFNγ stimulation has been shown to act on the type IV promoter of the *MHC2TA* gene, resulting in transcription of *MHC2TA type IV* (Dong et al., 1999).

A functional role for astrocytic MHC class II expression is supported by reports that rat astrocytes are capable of presenting myelin basic protein (MBP) on MHC II molecules to T cell lines (Fontana et al., 1984), but important biological effects of astrocytic MHC class II are contradicted by the low expression *in vivo*, even during inflammation (Hamo et al., 2007).

CNS antigens are thus presented on both MHC class I and II molecules, allowing interaction with infiltrating CD8+ and CD4+ T cells respectively. The key role of T cells in EAE is illustrated by the fact that EAE can be induced by transfer of CD4+ or, in irradiated recipients, by CD8+ T cells (Huseby et al., 2001). Increased numbers of T cells have also been observed in the injured CNS after experimental axotomy and spinal cord injury (Popovich et al., 1996; Raivich et al., 1998). In addition, mainly CD8+ T cells are present in the CNS during various human neurological conditions such as AD (Itagaki et al., 1988; Togo et al., 2002), traumatic brain injury (Holmin et al., 1998) and stroke (Dirnagl et al., 1999). In ALS, the presence of T cells is associated with motor neuron damage (Kawamata et al., 1992; Engelhardt et al., 1993). In MS, there is a profound cellular infiltration and active lesions are dominated by clonally expanded CD8+ T cells (Babbe et al., 2000). T cells could actually account for the axonal damage observed in MS, as CD8+ cells with polarized cytotoxic granules have been found in close proximity to injured axons in MS lesions (Medana et al., 2001).

2.4.3 Autoimmunity and neuroprotection

Autoimmunity is not synonymous with autoimmune disease, as also healthy individuals have circulating T lymphocytes specific for CNS antigens, and the number of myelin reactive T and B lymphocytes increase upon nerve injury (Olsson et al., 1993). Even manifested autoimmunity to CNS antigens may not solely have negative consequences. Studies show increased neuronal survival both in a rat optic nerve crush model after transfer of MBP reactive T cells (Moalem et al., 1999) and in VRA upon immunization with MBP (Hammarberg et al., 2000). In addition, immunization with MBP and transfer of MBP reactive T cells promoted recovery from spinal cord injury in the rat (Hauben et al., 2000). Induction of autoimmune encephalomyelitis also enhanced the survival of dopaminergic neurons in C57/Bl6 mice immunized with MOG_{35-55} in complete Freund's adjuvant (CFA) before chemically induced damage of the nigrostriatal dopaminergic system (Kurkowska-Jastrzebska et al., 2005). Immune challenge by CFA alone also increased neuronal survival, but to a lesser extent. A therapeutic potential of immunization is strengthened by the retained neuroprotective capacity of altered peptide ligands, which reduces the risk for pathogenic autoimmunity (Hauben et al., 2001).

Neuroprotection mediated by CNS-antigen specific effector T cells at an injury site can thus be induced by vaccination or by transfer. This protection could be attributed to lymphocytic production of neurotrophic factors. Production of neurotrophins have been reported in rat T cell lines (Moalem et al., 2000) and activated human T cells, B cells, and monocytes are capable of secreting bioactive BDNF *in vitro* (Kerschensteiner et al., 1999). In favor of neurotrophic effects during CNS disease, increased levels of BDNF transcripts has been found in the CSF of MS patients (Gielen et al., 2003).

In addition to neuroprotection via activation of autoimmune $CD4^+CD25^-$ effector T cells, downregulation of naturally occurring $CD4^+CD25^+$ regulatory T cells can have the similar neuroprotective effects (Kipnis et al., 2002). Bacterial DNA containing CpG motifs conferred neuroprotection after optic nerve injury by suppressing the activity of naturally occurring $CD4^+CD25^+$ regulatory T-cells (Johnson et al., 2007).

It has been suggested that the genetically determined susceptibility to autoimmune disease also determines the outcome after CNS injury (Kipnis et al., 2001). Rat and mouse strains that are resistant to EAE had twice as many surviving retinal ganglion cells after optic nerve injury. Autoimmunity resistant, but not susceptible, strains would thus mount a protective, T cell dependent response. This view is however contradicted by the outcome after spinal cord injury in the mouse (Basso et al., 2006; Kigerl et al., 2006) and rat (Birdsall Abrams et al., 2007), which was not correlated to the degree of EAE susceptibility.

In conclusion, the CNS is immuno-competent in many ways and the interplay between neurons, glia and systemically derived immune cells has an impact on both local processes such as neurodegeneration and on systemic responses. Typically, immunological processes in the CNS seem to have a dual role, with both beneficial and detrimental consequences. A thorough understanding of the nature of these processes is thus needed in order to modulate them for therapeutic purposes. Mapping the genetics behind altered susceptibility to CNS injury or disease is a strategy to identify both disease-promoting and protective targets.

3 AIMS OF THIS THESIS

The overall aim of this thesis was to investigate the genetic influence on neurodegeneration and inflammation in disease models under well controlled genetic settings.

- To perform a whole genome scan in search for QTLs regulating neurodegeneration, glial activation, MHC class II up regulation and T cell infiltration in the spinal cord after mechanical nerve injury.

- To fine-map QTLs in order to identify candidate genes regulating neurodegeneration and inflammation.

- To create congenic rats carrying neurodegeneration- and glial reactivity-associated QTLs for use in neurodegenerative disease models as well as in EAE.

- To extrapolate rat gene findings to human disease.

- To study the differences in global gene expression patterns in inbred rat strains after nerve injury.

4 METHODOLOGICAL CONSIDERATIONS

All materials and methods used are presented in detail in the respective papers. Here, specific considerations regarding the methods of choice, their advantages and limitations will be discussed.

4.1 ANIMAL MODELS

Animal experiments were approved and conducted according to the guidelines from the Swedish national board for laboratory animals and the European community council directive (86/609/EEC). Animals were bred in an in-house breeding facility with regular assessment of health status in the colonies.

Three intercrosses were studied in this thesis; F2(DAxPVG), G8(DAxPVG^{av1}) and G10(DAxPVG^{av1}). The AIL G8 and G10 populations were from the same descent, while the F2 was independent from these. The PVG founder strain in the F2 carried the original RT1^{c} MHC haplotype, while the AIL PVG^{av1} founder strain was congenic for the DA MHC haplotype, i.e. PVG.DA-*RT1*^{av1}. Intercrosses were initiated in a reciprocal fashion, with both DA and PVG (F2) or PVG^{av1} (AIL) females. This procedure was followed to account for parent-of-origin effects. Such effects were however not subjected to analysis in the studied cohorts.

Experiments including congenic rats aim at isolating QTLs to study their effect on selected phenotypes. A QTL that was identified in a heterogeneous population will thus be studied in a homogenous genetic setup. This is important to bear in mind when interpreting data obtained from congenic experiments, as both loss of and acquired epistatic effects could influence the results. The congenic strains included in Paper III were considered "pure" with a theoretical background contamination of <0.1% based on a 50% reduction in each BC generation. The true amount of donor DNA outside the congenic fragment was not estimated, and effects originating from introgressed alleles outside the selected congenic fragment can not be ruled out. To account for this, littermate controls were used to confirm the effect from *Vra4* on MHC class II expression in the CNS after nerve injury. For additional phenotypes such as EAE susceptibility, there is still a risk for effects from donor alleles outside the congenic fragment.

4.1.1 Experimental autoimmune encephalomyelitis

EAE induced in rats by immunization with rMOG in IFA results in MS-like pathology with focal lesions in the CNS, demyelination and production of rMOG-specific antibodies. The disease course varies from relapsing-remitting to chronic in DA rats (Weissert et al., 1998).

Weight loss is recorded and typically precedes clinical signs by 1-2 days. This will give a true quantitative phenotype with a normal distribution within the studied population. The clinical signs are assessed by the EAE score criteria where increased functional

loss is graded on an ordinal scale from 1-5 (Figure 6). This scale is not linear in nature, and resulting mean and cumulative score values will not be normally distributed. Non-parametric statistics are thus needed for analyses. Additional phenotypes used are the binary trait incidence and the ordinal trait day of disease onset. Although most EAE phenotypes are not true quantitative traits, they can be measured and used as such in QTL analyses.

4.1.2 Ventral root avulsion

In EAE, systemically induced inflammation will lead to systemic responses as well as local CNS pathology. This makes it difficult to isolate the impact of the target tissue, the CNS, on disease susceptibility and severity. For this purpose, other models are needed. VRA is a standardized, reproducible model for neurodegeneration and does not aim at resembling human disease but to feature specific processes related to neurodegeneration and local CNS inflammation. By avulsion of nerves originating at the lumbar segments L3-L5, large motoneuron populations innervating the hind limbs are targeted, facilitating neuronal counts (Figure 3).

By pulling the ventral roots, they will detach from the surface of the spinal cord, and minimize the amount of remaining nerve tissue. At dissection, a scar at the detachment site along the spinal cord can be clearly visible under the light microscope. This scar is evaluated by size, and protruding scar tissue removed before further dissection. Any bleeding or excessive scar formation is noted and evaluated for further analyses.

4.2 PHENOTYPES

4.2.1 Neuronal cell counts

Counting remaining nerve cells 2-3 weeks after nerve injury is a crude but relevant measure of the outcome after injury. The motoneuron loss is calculated as the number of motoneurons on the injured side compared to the intact side and is thus rather insensitive to inter-individual variations as well as variations depending on the specific anatomic location of the section. To get reliable results, several sections need to be counted for each individual hence we have calculated a mean survival ratio from a minimum of 15 sections. Due to injury-induced morphological changes, the mean soma diameter of remaining motoneurons on the injured side is around 72% of that on the intact side. To account for these differences, cell counts were adjusted according to the Abercrombie formula [N=n(T/(T+D))], where N=the true number of cells, n=the estimated number of cells, T=section thickness, and D=mean soma diameter of measured cells. Without correcting for soma diameter, the number of cells on the intact side will be overrated due to their relatively larger size. However, only motoneurons with a clearly visible nucleus were counted, reducing the true correction factor due to the smaller size of the nucleus compared to the soma diameter.

We have focused on the ratio of surviving motoneurons after injury, i.e. the end-point. Ideally, an easily measured predictive marker for the subsequent degree of motoneuron

loss could be used as a complement. Even if the determinant itself is not measured, it would allow for correlation of early injury responses to functional outcome. Up to date, no such predictive marker has been identified.

4.2.2 mRNA expression

Gene expression was analyzed by semi-quantitative reverse transcriptase- (RT-) PCR and by microarrays. Both techniques rely on extraction of total RNA but while RT-PCR is conducted on cDNA, Affymetrix microarrays are based on hybridization of cRNA to the chip. Degradation of RNA by RNases is an obvious risk, even though working under exogenous RNase-free conditions. RT-PCR primers are designed to amplify 5´sequences but the degree of RNA degradation is not quantitated. Since targets are related to internal controls, the relative concentrations could be considered constant if degradation occurs evenly. For Affymetrix microarrays, internal controls in the form of target sequences located in the 3´and 5´end of β-actin and glyceraldehyde-3-phosphate dehydrogenase (Gapd) are included. The ratio between the 3´/5´ signal values will thus give an estimate of the degree of RNA degradation in the sample, and must not exceed 2.

The relative quantification obtained by RT-PCR relies on the presence of internal controls in the form of housekeeping genes with assumed constant transcription and degradation rates in the studied samples. Relating the amount of target cDNA to the housekeeping gene cDNA will compensate for variations in sample size, the amount of extracted total RNA, the efficiency of cDNA synthesis and the degradation of RNA/cDNA. Any variation in the expression of the housekeeping gene between samples will, however, affect the estimated expression of the target. The use of complementary housekeeping genes is one way to reduce the risk for such effects, and was employed in Paper IV. In Paper IV, 18S rRNA was used as an additional reference. The use of rRNA as internal control is similar to that of housekeeping gene mRNA, with the exception that poly-dT primers may not be used for reverse transcription. If an internal control can not be used, the amount of target can be related to the sample size or the initial total RNA amount, but this will not control for errors introduced during RNA and cDNA preparation or PCR. Alternatively, an introduced molecule of known concentration can be used as reference (Huggett et al., 2005).

4.2.3 Protein expression

Gene expression analysis by measuring mRNA has the limitations of being an intermediate phenotype. A relative higher amount of mRNA is likely to reflect a higher amount of protein, but this is not always the case. Therefore, protein expression can be studied as the end-product of gene transcription. Protein expression analyses by use of immunostainings can also measure post-translational quantitative effects like protein turnover due to the stability and degradation rate. Additional information from protein stainings include cellular localization and the cell type and anatomical localization of positively stained cells.

4.2.4 *Ex vivo* studies

In order to manipulate a specific cell population, it can be isolated and studied *ex vivo*. In Paper II, peripheral blood cells were extracted from whole blood samples from rheumatoid arthritis (RA) patients selected on the basis of their *MHC2TA* genotype. Cells were stimulated with IFNγ at increasing concentrations for 6 hours. IFNγ is a known regulator of MHC2TA and MHC class II expression and was selected to study genotype-associated expression differences of these genes. The setup is purely experimental as no antigen presentation, priming or restimulation will occur. Expression differences between the *MHC2TA* genotype groups were more pronounced when increasing the stimulation by IFNγ. This may be due to overriding inter-individual effects due to other genes and environmental factors. The simplified *ex vivo* system thus gives clues on the capacity of cells with a specific *MHC2TA* genotype to induce MHC class II expression, but the mechanisms in disease have to be studied further.

4.3 STATISTICS

Student's t-test was used to compare two groups with assumed normally distributed phenotypes. Non-parametric tests were used for phenotypes assumed not to follow a normal distribution but had a total sample size >7. Expression data obtained by RT-PCR and the motoneuron survival phenotype are both calculated as ratios and thus treated as non-parametric. Non-paired observations (e.g. expression of a target in two independent groups) were analyzed by the Mann-Whitney rank sum test while comparisons of three or more groups were done by Kruskall-Wallis analysis of variance followed by Dunn's post test.

Correlation in expression of two targets was analyzed with non-parametric Spearman rank test, which provides an r- and a p-value. The r value quantifies the direction and magnitude of correlation between X and Y, while the p-value is a measure of the likelihood of the obtained r value.

4.3.1 Linkage in experimental populations

The whole genome scan in F2 and the fine-mapping of *Vra4* in G8 were performed by linkage analysis in MAPMAKER/QTL (Lander et al., 1987; Lander and Botstein, 1989) combined with R/QTL (Broman et al., 2003).

Linkage by interval mapping is based on the presence of a genetic map and tests the position of a QTL along every given position of the map. The genetic map is measured in Morgan, or cM. One M (100 cM) equals a recombination frequency of one per meiosis. To calculate the genetic map, the maximum likelihood method is used. This is a method that extends the applications of counting recombinants, since it also accounts for heterozygotes. The likelihood of obtaining the observed results given a specific map is calculated and compared to alternative maps. The map giving the highest likelihood will then be the best estimate and be used for QTL positioning. MAPMAKER/EXP

uses an algorithm developed by Lander and Green for the creation of genetic maps (Lander and Green, 1987).

Interval mapping was introduced by Lander and Botsein in 1989 (Lander and Botstein, 1989). Originally, algorithms were developed for normally distributed traits. Some, but not all, data can be transformed in order to obtain normality and use parametric tests. In 1995, MAPMAKER/QTL was complemented with a version of the rank-sum test, thus allowing interval mapping of non-parametric traits (Kruglyak and Lander, 1995). For the phenotypes studied in Paper I and II, data for neuronal survival and MHC class II immunolabeling were subjected to log (base 10) transformation to obtain more symmetric distributions.

In order to evaluate data from interval mapping, appropriate significance thresholds have to be set to judge if a QTL is significant, suggestive or random. Significance thresholds for QTLs have to take multiple testing into account in order to reduce the false positive rate. Lander and Botstein related the LOD score to a known random process to obtain appropriate thresholds (Lander and Botstein, 1989). The R/QTL software has the advantage of setting data set specific thresholds. Permutations (10.000) were performed in R/QTL to generate data set specific significance levels in Paper I and II (Churchill and Doerge, 1994). In addition to setting significance thresholds, the R/QTL package includes estimation of genetic maps, identification of genotype errors and inclusion of covariates. Since both single-QTL and two-QTL scans can be performed, interaction analyses can be performed (Broman et al., 2003). The alternative methods used in R/QTL include the EM algorithm used by MAPMAKER (Lander and Botstein, 1989), Haley-Knott regression (Haley and Knott, 1992), multiple imputation (Sen and Churchill, 2001) and nonparametric interval mapping.

To further investigate a significant QTL, a location CI has to be set. This CI can not tell the position of the QTL, but its boundaries represent the probability (e.g. 0.95) of containing the true location. In theory, a saturated genetic map will give a CI estimate that is the inverse function of the sample size, the number of informative meioses per individual and the squared function of the QTL effect (Darvasi and Soller, 1997).

Lander and Botstein suggested estimation of "support intervals" for QTL location based on the likelihood ratio test (Lander and Botstein, 1989). This is based on the likelihood maximized over all parameters compared with the maximum likelihood when some parameters are fixed. Simulations can also be used for CI estimates. Non-parametric bootstrapping is based on sampling with repeats from the original data. Many artificial data sets of size equal to the original set are thus generated, with random representation of each sample from the original set. The CI estimate is then determined by the distribution of artificial CI positions. This method performs well when QTLs do not exceed 2/3 the size of the chromosome (Visscher et al., 1996). However, the accuracy of non-parametric bootstrap for QTL location has been questioned by the fact that LOD curves tend to peak at genetic marker locations, thus affecting the CI estimate by bootstrapping (Manichaikul et al., 2006). Alternatives are Baye's credible intervals, based on posterior probability, or the classical likelihood support interval. In Paper I and II, a likelihood support interval of 1.5 LOD drop was employed to estimate CIs. This was proposed by Dupuis and Siegmund to obtain 95%

coverage of a dense marker map (Dupuis and Siegmund, 1999). The coverage of a LOD support interval is mostly affected by marker spacing and the QTL effect, with smaller influence by the sample size. A smaller drop is needed with dense markers and large QTL effects. A LOD support interval of 1.8 was suggested for intercrosses with down to 1 cM marker spacing (Manichaikul et al., 2006).

4.3.2 Human association studies

When selecting markers to be included in association analysis, certain criteria should be fulfilled: The markers should be in Hardy-Weinberg equilibrium and have a genotype frequency close to that predicted by the minor allele frequency (MAF), the MAF should not be too low (<0.2), or the power of the analysis will be reduced and importantly, the markers should perform well in the genotype assays used.

In paper II, three SNP genotyping methods were used: the 5' nuclease assay (Livak, 1999), dynamic allele-specific hybridization (DASH) (Jobs et al., 2003) and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Jurinke et al., 2002). The 5' nuclease assay is a TaqMan based technique where fluorescently labeled allele-specific probes bind their targets and are cleaved by the Taq DNA polymerase. In DASH, labeled probes are hybridized to PCR products bound to a membrane. The resulting melting curves will then discriminate between probe-matched and probe-mismatched targets. MALDI-TOF is a mass spectrometry method where oligonucleotides are ionized and accelerated in an electric field that will separate them according to their mass-to-charge ratio.

Depending on the biological effect of an analyzed marker or the causative polymorphism linked to the marker, different models for analysis are used. In the codominant model, all three genotype groups are analyzed, while in the dominant model, heterozygotes are included in the dominant allele homozygous group. Allele frequencies may also be directly compared and the analysis will thus not discriminate between heterozygotic and homozygotic carriers.

The identification and analysis of haplotype blocks may increase the power to detect association. A haplotype has a higher chance of catching the causative polymorphism, even if not typed, as information from several markers is used to track its effects. In 2005, the international HapMap project published a haplotype map of the human genome based on a million SNPs in four different populations; European, Japanese, Chinese Han and African Yoruba (International HapMap Consortium, 2005). The haplotype map is constantly being updated and is now of the 22nd release. An advantage of the haplotype map is that a few "tag" SNPs can be used to cover most of the variation in a region, thus reducing the number of SNPs to genotype.

5 RESULTS AND DISCUSSION

5.1 WHOLE GENOME SCAN OF VRA RESPONSES (PAPER I)

The genetic regulation of neurodegeneration and local inflammation after VRA was previously reported from data based on inbred rat strains. Hence, different strains showed a distinct pattern of VRA responses, and data from MHC-congenic strains showed that the responsible genes for the studied phenotypes mainly reside outside the MHC itself (Piehl et al., 1999; Lundberg et al., 2001). To position gene regions affecting the different aspects of the VRA response, an intercross between the DA strain, displaying a phenotype with pronounced motoneuron loss and local inflammation, and the PVG strain, that lose fewer neurons and display a lower degree of local inflammation, was performed. These strains carry the $RT1^{av1}$ and $RT1^{c}$ MHC haplotype, respectively, allowing mapping of effects from the MHC. A total of 186 animals in the F2 generation were subjected to VRA and neuronal loss, T cell infiltration, MHC class II up regulation and glial activation were assessed 14 days later. Genotyping was performed with evenly spaced microsatellite markers across the rat genome to allow a whole-genome scan for each phenotype.

5.1.1 *Vra1-4*

Whole genome scans in the F2 population identified four different QTLs linked to MHC class II expression, neurodegeneration and/or T cell infiltration (Table 1). Glial activation as measured by microglial expression of CD11b/c and astrocytic up regulation of GFAP did not display linkage to any specific gene region, although the parental strains differed significantly in both parameters. The degree of neurodegeneration was measured as the motoneuron survival i.e. the ratio of remaining motoneurons on the injured side compared to the intact side. Motoneuron survival was 36% higher in PVG compared to DA rats. Although a complex and prolonged process in nature, the genetic determinants for the degree of neurodegeneration in this strain combination resolved into only one significant, *Vra1*, and one suggestive, *Vra2*, QTL. The latter was also linked to the number of infiltrating (CD3+) T cells, a phenotype to which a third QTL, *Vra3*, displayed suggestive linkage.

Table 1. Summary of QTLs identified in the DAxPVG F2 whole genome scan. *Suggestive linkage. Positions (RNO:Mb) are given in for markers flanking a LOD support interval of 1.5.

QTL	Phenotype	Flanking markers	Position	Size (Mb)	Max marker	LOD
Vra1	Neurodegeneration	D8Rat99-D8Rat129	8: 43.56 - 98.95	55.4	D8Rat205	5.5
Vra2*	Neurodegeneration	D5Rat73-D5Rat82	5: 25.60 - 45.82	20.2	D5Rat70	3.6
Vra2	T cells	D5Rat125-D5rat82	5: 17.70 - 45.82	28.1	D5Rat70	4.6
Vra3*	T cells	D5Rat178-	5: 161.45 - end	11.7	D5Rat50	3.4
Vra4	MHC class II	-D10Rat218	10: start - 9.09	9.1	D10Rat95	27.4

For MHC class II expression, only *Vra4* at the acrocentric part of chromosome 10 was significant, with an extremely high LOD score of 27.4. The MHC located on chromosome 20 was, however, found to reach significance when stratifying the data for the *Vra4* candidate gene (*Mhc2ta*) allele in a later analysis (Figure 7). This effect thus seems to have been masked by the strong impact of *Vra4* in the initial analysis. No interactive effect was seen between *Vra4* and the MHC arguing for a relatively small effect in this genetic context. The possible biological importance in an outbred population can however not be assessed by this data.

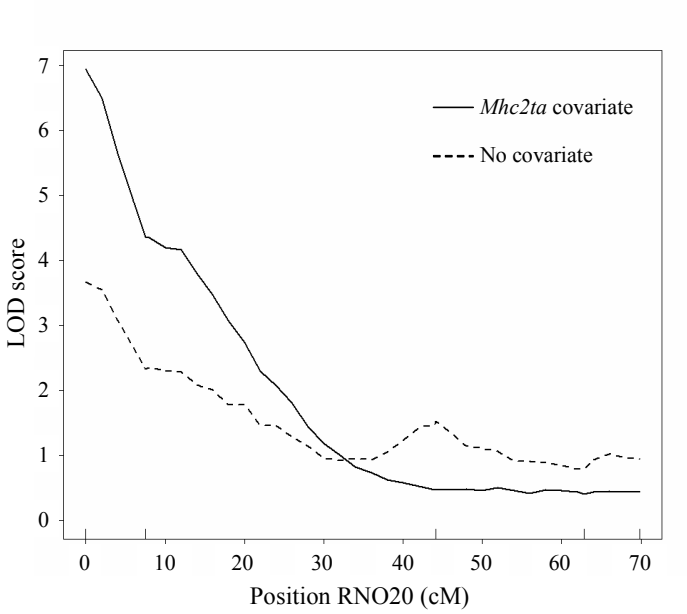

Figure 7. Linkage of MHC II expression to RNO20 with (solid line) and without (dashed line) *Mhc2ta* gentoype as covariate. The max marker D20Rat41 (LOD 6.9) is situated in the MHC at 0 cM. The CI estimated as a LOD support interval of 1.5 is flanked by the marker D20Mgh5 at 8 cM.

5.1.2 Interconnected phenotypes

The presence of CD3+ cells in the ventral horn of the spinal cord after VRA probably reflects infiltrating T cells, as described by others under neurodegenerative conditions (Itagaki et al., 1988; Kawamata et al., 1992; Engelhardt et al., 1993; Raivich et al., 1998; Togo et al., 2002). T lymphocytes have been reported to exert both protective (Moalem et al., 1999; Hammarberg et al., 2000) and neurotoxic effects on injured neurons (Kawamata et al., 1992; Engelhardt et al., 1993; Medana et al., 2001). The correlation between CD3+ cells and degree of neuronal survival (Figure 8) (r=0.21, p=0.007) and the linkage of both CD3+ cell numbers and neurodegeneration to *Vra2* indicate a role for the extent of infiltrating lymphocytes in the neurodegenerative process. Increased CD3+ cell numbers could either be causative or a consequence to increased cell death. *Vra2* may also harbor independent QTLs for these phenotypes. Due to the low resolution in an F2 scan, such closely linked QTLs are not possible to discriminate between.

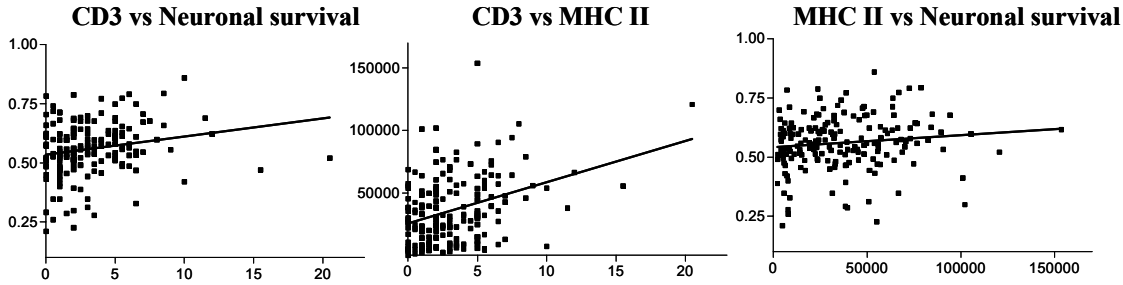

Figure 8. Correlations between phenotypes in the DAxPVG F2 data set. The number of CD3+ cells was significantly correlated to neuronal survival (r=0.21, p=0.007) and MHC class II expression (r=0.30, p<0.001). No significant correlation was seen between MHC class II expression and neuronal survival.

The number of CD3+ cells was also correlated to the degree of MHC class II expression (Figure 8) (r=0.30, p<0.001). According to immunolabeling, 40% of the CD3+ cells were CD4+, allowing interaction with MHC class II molecules on microglia. One could speculate that an increased expression of MHC class II would enable retention and activation of infiltrating CD3+/CD4+ T lymphocytes. However, any phenotype being a straight consequence of another phenotype would by default be linked to the same QTL. The lack of a common QTL for the phenotypes CD3+ cell numbers and MHC class II expression thus argues against a strong connection.

No correlation was observed between the two phenotypes MHC class II expression and neuronal survival (Figure 8). This agrees with findings in inbred rat strains, where the E3 strain display very sparse expression of MHC class II after VRA but a pronounced neurodegeneration (Lundberg et al., 2001).

5.2 FINE-MAPPING OF *VRA4* (PAPER II)

The whole genome scan performed in a DAxPVG F2 intercross identified one locus, *Vra4*, with a very strong linkage to MHC class II expression (LOD 27.4, Paper I). The likelihood support interval of 1.5 LOD drop spanned approximately 4 cM, corresponding to a 9.1 Megabase (Mb) region at the acrocentric part of RNO10. To position a candidate gene in the region and enable association analysis in human disease, *Vra4* was fine-mapped by use of an AIL between DA and PVG^{av1} rats, following the same experimental VRA setup as for the F2 cross, and measuring MHC class II expression in the injured ventral horn at 14 days after VRA. The human orthologue to the identified gene, *MHC2TA*, was subsequently tested for association to RA, MS and MI.

5.2.1 Candidate gene identification

Interval mapping in the G8 generation of a DAxPVG ^{av1} AIL confirmed *Vra4* (LOD 26.4) and narrowed the region to a 5.3 Mb region at the very acrocentric part of RNO10. The reduction of the CI (40%) is less than what would be expected at the G8 generation (75%). This could be due to the acrocentric position of the QTL at the very beginning of RNO10, giving both an asymmetric QTL LOD curve and relatively few recombination events. The G8 CI contained around 40 genes, still a too large number for functional studies. We first aimed at minimizing the gene region, then to study candidate genes within the minimal fragment (Figure 9). As a first step a haplotype map based on SSLPs in six inbred rat strains with phenotypic data on MHC class II expression was created. If the observed difference in MHC class II expression in the rat strains included in the haplotype analysis was a result of a common inherited allelic variant, this could theoretically be tracked by the more polymorphic SSLPs surrounding the candidate gene. The haplotype map identified a 2.7 Mb large haplotype region segregating rat strains into high and low MHC class II expressers. The 23 genes annotated in mouse or rat in this region were reduced to 13 by analyzing recombinant AIL rats from the G10 generation. The phenotype in these rats was attributed to a 0.9 Mb region between markers D10Mgh25 and D10Mco46. Sequencing and expression analysis of candidate genes within the recombination interval pinpointed *Mhc2ta* as the candidate gene.

The syntenic human gene, *MHC2TA,* was first described in the hereditary human disease bare lymphocyte syndrome, a severe MHC class II deficiency caused by a null mutation in *MHC2TA* (Steimle et al., 1993). An extensive literature describes the gene product, CIITA, as the global regulator of both constitutive and inducible MHC class II expression (Chang and Flavell, 1995; Kern et al., 1995; Chang et al., 1996; Muhlethaler-Mottet et al., 1997; Nagarajan et al., 2002; Ting and Trowsdale, 2002). There were however no prior reports of functional genetic variants of *MHC2TA*, instead such were considered to have a too large impact on immune responses to be conserved in the population (Janitz et al., 2001).

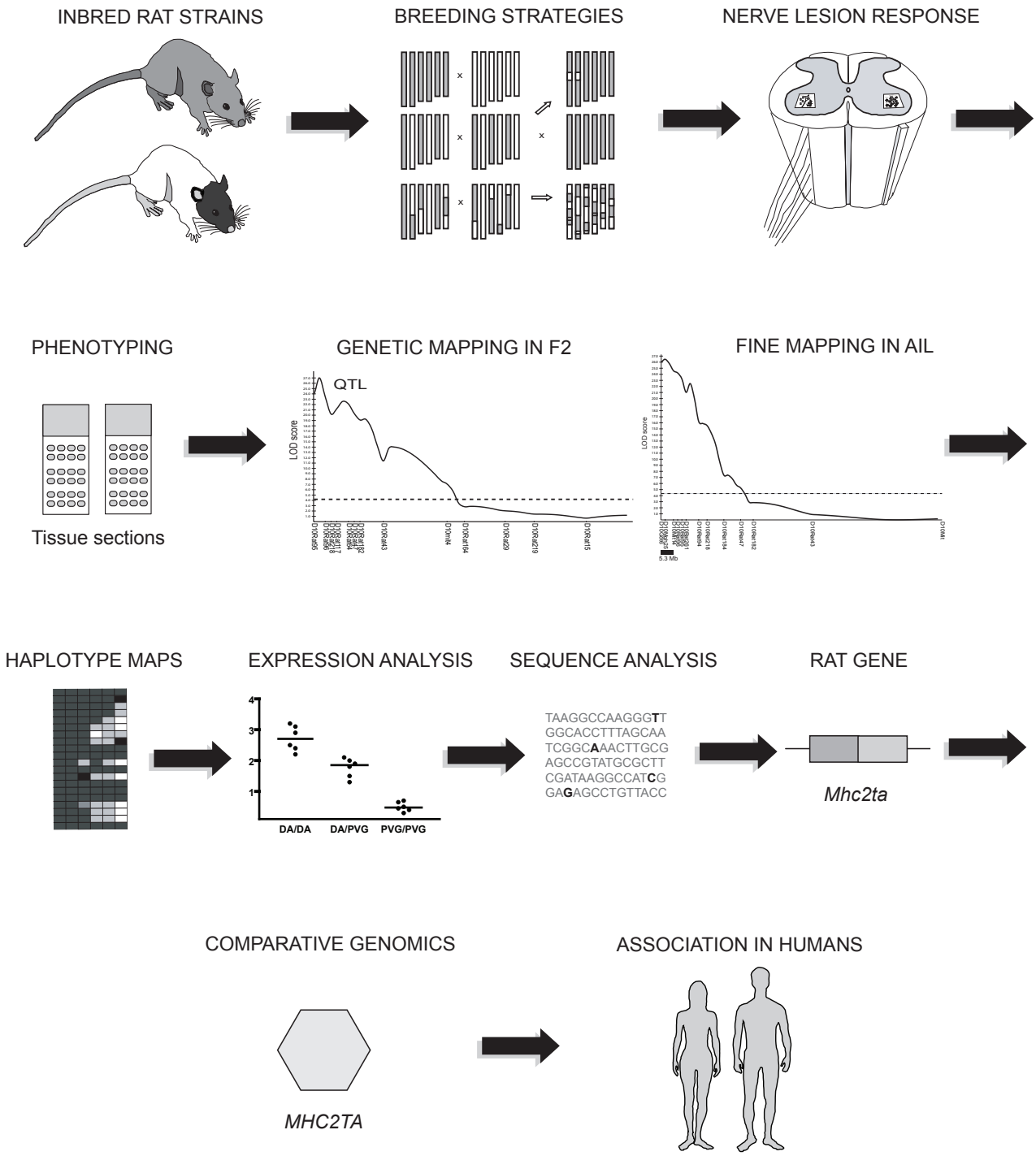

Figure 9. Strategy to identify *Mhc2ta* as the candidate gene for *Vra4* and for comparative genomic studies in humans. DA and PVG rats were intercrossed to produce F2 and AIL generations. A whole genome scan identified *Vra4* with very strong linkage to upregulation of MHC II expression after VRA. Fine mapping in AIL and a haplotype map helped to narrow *Vra4*. Expression analysis combined with sequencing identified *Mhc2ta* as the candidate gene. The corresponding human gene, *MHC2TA* was subsequently tested for association to three inflammatory diseases. Modified from Piehl et al, 2007.

5.2.2 Human association

Based on the finding that functional polymorphisms in the 5´region of *Mhc2ta* modulate MHC class II expression in the rat CNS, we selected three human diseases with inflammatory components for association analysis of the corresponding gene, *MHC2TA*. Both RA and MS are chronic, inflammatory disorders (reviewed in (Klareskog et al., 2006) and (Hafler et al., 2005)) and there are inflammatory processes of atherosclerosis and myocardial infarction (MI) (Libby, 2002). Seven SNPs in the *MHC2TA* region were analyzed and association analyses were based on three SNPs; SNP1 (rs3087456, -168A/G) located in the 5´flanking region of the *MHC2TA* type III promoter (Patarroyo et al., 2002) and SNP2 (rs2229320plus27bp) and SNP3 (rs4774) located in exon 11. The association to disease was not improved by haplotype analysis but could be attributed to a dominant effect of the G allele at -168A/G alone (Table 2). Whether -168G itself is responsible or is linked to the causative polymorphism can not be outlined by this analysis. It is interesting to note, however, the concordance with the findings in the rat with two SNPs in the type III promoter segregating with degree of MHC class II expression. Recent data from the corresponding mouse gene, *C2ta*, shows the same pattern with polymorphisms in the type III promoter segregating high- and low expressing strains (Harnesk et al, unpublished observations). The location of the human -168A/G SNP in upstream, regulatory regions of *MHC2TA* indicates that the functional polymorphism linked to -168G (or -168G itself) affects transcription. To identify the functional polymorphism(s) is challenging. With the ultimate goal of modifying disease, a focus on the downstream biological effects may instead be more favorable.

When using the corresponding control groups for each disease, -168G was found to be significantly associated to RA and MI, but not to MS (Table 2). Association to MS was however seen when using the population-based RA controls as well as all combined controls. The MS controls were healthy blood donors, and the exclusion criteria for listing as blood donor, e.g. allergy, medication and chronic disease may have skewed this group from the allele frequencies in the normal population. The -168 AG/GG genotype frequency in the population-based RA controls was very similar to what was observed in a large control material from a later study in the Swedish and Finnish populations (36% compared to 37%) (Lindholm et al., 2006).

Table 2. Association according to G dominant model for the *MHC2TA* -168 A/G SNP (rs3087456) in MI, MS and RA materials. P values are given for χ^2 test and ORs are given for 95% CI.

			AG+GG	Respective control group		All controls	
	AA	AG+GG	frequency	p	OR	p	OR
MI	199	177	0.47	0.025	1.39 (1.04-1.85)	0.002	1.44 (1.15-1.80)
MI controls	236	151	0.39				
MS	295	225	0.43	0.311	1.14 (0.89-1.46)	0.038	1.24 (1.01-1.51)
MS controls	304	204	0.40				
RA	728	534	0.42	0.008	1.29 (1.07-1.56)	0.024	1.19 (1.02-1.38)
RA controls	449	255	0.36				
All controls	989	610	0.38				

The positive association in independent materials strongly supports a functional role for *MHC2TA* polymorphisms in complex inflammatory diseases. The relatively weak association and low ORs, however, make follow-up studies important to evaluate these results (see general discussion).

5.2.3 Functional studies

As a functional assay on the degree of MHC class II up regulation after inflammatory stimulus, we employed an *ex vivo* stimulation of peripheral blood cells (PBCs) with increasing concentrations of IFNγ. Blood was sampled from RA patients with the -168 A/A, A/G or G/G genotype. Expression of *MHC2TA*, *CD74*, and the HLA genes *DQA1* and *DRA* was measured by semi-quantitative RT-PCR and related to the housekeeping gene *GAPD*. Upon stimulation with IFNγ, PBCs with the G/G genotype showed a significantly lower induction of *MHC2TA*, *CD74* and HLA transcript expression compared to the combined A/G and A/A group, and the differences increased with stronger stimulation. In addition, *MHC2TA* transcript levels were highly correlated to the transcript levels of all three MHC-associated transcripts in individual samples.

The finding that the risk genotype G/G at -168A/G corresponded to low MHC class II expression *ex vivo* is intriguing. In the rat, the low-MHC (and low *Mhc2ta*-) expressing strain PVG^{av1} is less susceptible to both experimental arthritis and EAE compared to the DA strain (Lorentzen and Klareskog, 1996; Storch et al., 1998; Weissert et al., 1998). The simplified stimulation model used *ex vivo* may not be biologically relevant for the complex interplay between immune cells and molecules *in vivo*, especially during disease pathogenesis. Alternatively, the consequences of genetic heterogeneity in *Mhc2ta/MHC2TA* vary between species. If the human disease-associated G/G genotype is indeed correlated with lower expression of MHC class II on PBCs *in vivo*, this could have impact on the Th1/Th2 balance and thus the regulatory effect of T cells. A role for regulatory T cells has been proposed in all three diseases studied here (Cao et al., 2003; Mallat et al., 2003; Viglietta et al., 2004).

5.3 IMMUNOMODULATION BY *VRA4* (PAPER III)

In order to study the biological effects of the *Vra4* QTL and its candidate gene *Mhc2ta*, reciprocal congenic rats were bred by transferring the *Vra4* locus from PVG^{av1} to DA, creating the DA.PVG^{av1}-*Vra4* congenic strain, and vice versa (*Vra4* locus from DA to PVG^{av1}) for the PVG^{av1}.DA-*Vra4* congenic strain (see Figure 2). Littermate controls (lm) i.e. siblings to congenic rats that lack the congenic fragment and were obtained during homozygotization were included in the first experiments to assess the effect of contaminating donor genome outside the *Vra4* locus. Theoretically, this contamination is less than 0.1%, corresponding to about 20 genes. Since the lm rats are >99.9% genetically identical to the parental strain, they were called DA lm and PVG^{av1} lm respectively. Congenic rats were subjected to VRA in order to assess the degree of microglial MHC class II expression after nerve injury. Intraparenchymal injections of IFNγ were administered to study the response to classic inflammatory stimulus. Finally, congenics and parental strains were immunized with rMOG in IFA to evaluate the effect of *Vra4* on susceptibility to EAE.

5.3.1 VRA

DA, DA lm, PVG^{av1}, PVG^{av1} lm, DA.PVG^{av1}-*Vra4* and PVG^{av1}.DA-*Vra4* rats were subjected to VRA and sacrificed 21 days later. Spinal cord was dissected and ventral quadrants from the L3 segments were used for RNA extraction while L4 segments were sectioned and used for immunohistochemical labeling.

Transcript levels of *Mhc2ta* and *Cd74* as well as protein expression of MHC class II could be attributed to the *Vra4* origin in both the reciprocal congenics. The phenotypes thus reflected that of the *Vra4* donor strain, while no differences were seen between the parental strains and their corresponding lm controls. One exception was expression of *Mhc2ta* and *Cd74* in the PVG^{av1}.DA-*Vra4* congenic, which was even higher than in the DA strain. Such a tendency was seen also in a pilot study in the 6th BC generation of this strain. A possible explanation could be interactions or allowance of penetrance of PVG^{av1} alleles in the congene background genome when exchanging the *Vra4* locus. A similar effect was recently found in another strain combination in the lab, where a F2 generation between the BN and LEW^{n} rat strains was studied with regard to early MHC class II upregulation after nerve injury. These strains share the same *Mhc2ta* haplotype but the LEW^{n} strain displays a slower and lower MHC class II expression compared to the BN strain. In spite of this, a QTL on chromosome 7 for MHC class II expression was found to be LEW^{n}-driven, resulting in higher expression of MHC class II in F2 individuals carrying LEW^{n} alleles at this locus as compared to BN alleles (Diez, unpublished). The situation in an intercross where two parental genomes are randomly mixed is very different from that in the parental strains, since genetic recombinations will occur allowing for new allelic interactions (reviewed in (Wandstrat and Wakeland, 2001)). Examples of epistatic effects where alleles from a more resistant parental strain result in increased disease severity when interacting with new alleles at another locus include pituitary tumor growth (Wendell and Gorski, 1997), pristane-induced arthritis (Lu et al., 2002), and EAE (Jagodic et al., 2005). Although limited to specific genomic regions, similar epistatic effects could be found in congenic strains (Monti et al., 2003).

5.3.2 IFNγ injections

To study the responsiveness of microglial cells to classic inflammatory stimuli, we performed intraparenchymal stereotactic injections of IFNγ in DA and DA.PVG^{av1}-*Vra4* congenics. Three days later, there was a strong up regulation of MHC class II in the DA rat brains, while the DA.PVG^{av1}-*Vra4* congenics showed a much lower MHC class II expression. These results are in accordance to the findings in parental strains (Paper II) and show that allelic differences in *Vra4* have impact on MHC class II expression in different settings, including classic inflammatory stimulus.

5.3.3 EAE

Expression of the mouse class II transactivator (*C2ta*) gene isoforms I and IV has been demonstrated in the CNS of mice with EAE (Suter et al., 2000), but the impact of *C2ta* on disease has previously been limited to studies in knock-out and transgenic mice. Knock-out mice for *C2ta* have been reported to have impaired MHC class II expression, with the exception of nonconventional MHC class II and MHC class II on a subset of thymic epithelial cells (Chang et al., 1996). As a consequence, very few mature CD4+ T cells were present in the periphery of these mice. Furthermore, studies on EAE in *C2ta* knock-out mice showed that they retained the capacity for encephalitogenic antigen presentation to CD4+ T cells in the periphery but were resistant to induction of EAE both by active immunization and by adoptive transfer of encephalitogenic T cells (Stuve et al., 2002; Tompkins et al., 2002). Targeted gene expression of *C2ta* in astrocytes of transgenic mice with *C2ta* under control of the GFAP promoter did not affect the resistance to EAE (Stuve et al., 2002). The studies on *C2ta* deficient mice clearly indicate a role for *C2ta* in the initiation and propagation of an encephalitogenic process, but have the disadvantages of knock-out studies regarding loss of function. Instead, to evaluate the clinical effect of functional allelic variants of a gene, congenic animals are more suitable.

In a first experiment, male and female rats from each congenic and the parental strains were immunized with mild to moderate doses of rMOG in IFA. Both the PVG^{av1} and PVG^{av1}.DA-*Vra4* rats were resistant to disease while the DA and DA.PVG^{av1}-*Vra4* strains developed a rather mild EAE. The DA.PVG^{av1}-*Vra4* congenics were protected from clinical signs of EAE in a dose-dependent manner, where the protective effect was best seen in congenic males receiving the higher dose (80 μg) and in congenic females receiving the lower dose (60 μg) of rMOG. In contrast, there was no difference between female DA and congenic rats receiving the higher rMOG dose, and disease severity was to low to evaluate the effect on males receiving the low rMOG dose. The different optimal doses for the two sexes probably depend on the fact that male rats are bigger compared to females at the same age and both weight and sex are parameters that affect the susceptibility to disease induction. It is interesting to note that the effect of *Vra4*-mediated protection to EAE clearly depends on the degree of challenge. In a complex disease like MS, several genes and environmental factors together make up an individual's susceptibility to disease. The genetic influence of a specific gene, in this context the *MHC2TA*, may thus be penetrant or not depending on the combined load of susceptibility versus protective factors (see Figure 1). In the simplified rat model used

here, this balance mainly depends on the immunization protocol, sex and size of the animal.

Based on the first study, female DA and DA.PVG^{av1}-*Vra4* congenics (n=15) were immunized with 10 μg of rMOG in IFA. The lower dose in this experiment was derived from a second batch of rMOG and chosen to induce a rather mild disease according to previous titration experiments. The congenics were significantly protected from EAE with both a lower incidence, i.e. lower susceptibility, and a lower cumulative score, i.e. lower disease severity compared to the DA strain.

The *Vra4* locus isolated in reciprocal congenic rat strains thus changed the parental phenotype of MHC class II-associated gene and protein expression to that of the *Vra4* allelic origin. In addition, *Vra4* alleles from the PVG^{av1} strain mediated significant protection to rMOG induced EAE when transferred to the EAE susceptible DA background, creating the DA.PVG^{av1}-*Vra4* congenic strain.

5.4 GLOBAL GENE EXPRESSION AFTER VRA (PAPER IV)

Earlier studies, where a panel of inbred rat strains was subjected to VRA, demonstrated significant strain-dependent differences with regard to degree of motoneuron loss as well as local inflammatory responses (Piehl et al., 1999; Lundberg et al., 2001). The genetic effect on the phenotypic parameters studies could be mainly attributed non-MHC genes (see Figure 5). Of the strains studied, the DA and PVG strains represented the two extremes both for neurodegeneration and inflammation and were selected for linkage analysis (paper I and II). To characterize the differences in gene expression preceding and during the response to nerve injury between these strains, the Affymetrix microarray technology was employed. Spinal cord tissue was sampled before, 5 and 14 days after VRA, the two latter time points representing events prior to, and at the midst of the neurodegenerative process, respectively (see Figure 4).

For each strain, a time point was represented by three microarray chips hybridized to independent pools of cRNA from four individual rats. This setup allowed both an analysis of the kinetics of the VRA response in each strain and a strain comparison at each respective time point (Figure 10).

Of the >8000 investigated sequences, in average 45% were expressed in the pooled samples. Among these, we found 278 regulated genes, where 245 were regulated during the VRA response and 68 were strain regulated at one or more time points. The overlap between the two analyses was 35 genes (Figure 11). These 35 genes clearly regulated during the response to nerve injury and differing between the two strains are obvious candidates for further studies combining expression and QTL data.

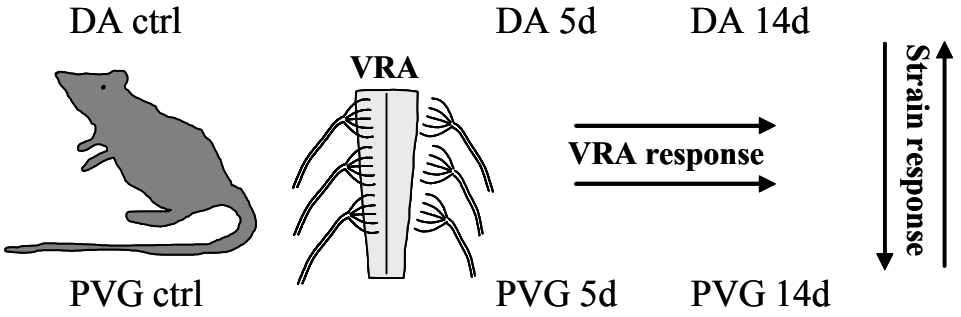

Figure 10. Experimental setup of the global gene expression study. Three microarrays respresent each respective strain and timepoint. To study the VRA response, comparisons were made within a strain with naïve animals (ctrl) as baseline. To study the strain response, strains were compared at each timepoint.

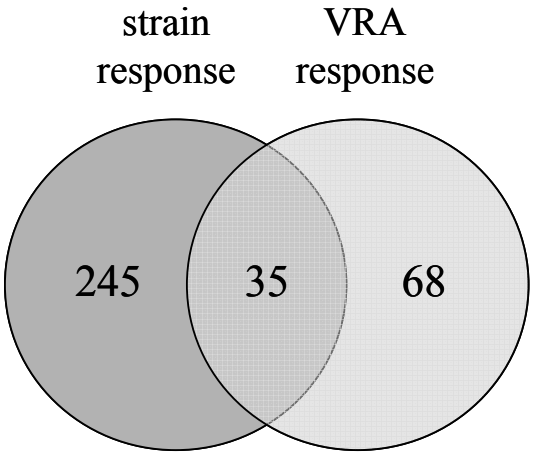

Figure 11. Overlap between strain- and VRA-regulated genes. In total, 278 genes were regulated by strain and/or injury.

5.4.1 Kinetics of the VRA response

Comparing the six groups in the experiment, principal component analysis (PCA) segregated the groups primarily by injury. According to the PCA, the injury response accounted for 56.2% of the variability between groups. This represents the common injury response in DA and PVG rats to VRA. There was a clear pattern in the regulated genes after VRA with all the 41 regulated genes classified to the immunology and inflammation group were up regulated, and a majority (31) of the 41 regulated genes in the neurotransmission group were down regulated.

Some of the VRA regulated genes have been reported previously. A cDNA microarray gene expression study of outbred Sprague Dawley (SD) rats at 3 days after injury showed an up regulation of matrix metalloproteinases *Mmp2*, *3*, *7* and *16* and the tissue inhibitor of metalloproteinases (*Timp*) *1* and *2* (Hu et al., 2002). Our results confirm VRA regulation of *Mmp3*, *16* and *Timp1*. The synaptosomal associated gene transcripts *Snap25* and *Sv2b* were down regulated, consistent with earlier microarray studies (Hu et al., 2002) (Yang et al., 2006) and descriptive reports (Jacobsson et al., 1996) on spinal root avulsion. This may reflect loss of synaptic terminals and abnormal transmitter release.

5.4.2 Strain differences

The second most prominent factor separating the six studied groups was strain. In the PCA analysis, the strain response accounted for 28.1% of the variability. Strain differences in gene expression patterns after CNS injury have not been extensively studied so far. Minor strain differences between SD and athymic nude rats (2% of the regulated genes) were reported in a spinal cord contusion model (Velardo et al., 2004). The SD rats are outbred which make an estimate of the genetic effect on transcript levels difficult from these results. In our study, 24% of the regulated transcripts were differentially regulated between the inbred DA and PVG strains before and/or after injury. Significant differences in gene expression between rat strains differing in

complex traits is supported by Kimpel and coworkers, who studied the gene expression patterns in five different brain regions of alcohol-preferring and non-preferring naïve rats. In total, 296 genes were differentially expressed between the two strains and 71 were found to be localized to established rat alcohol QTLs (Kimpel et al., 2007).

Our results show a correlation with neurodegeneration for the strain regulated genes *Timp1* and the complement component *C1qb*. *Timp1* has, as mentioned above, consistently been shown to be regulated after nerve injury. Complement components have been reported to be up regulated in peripheral nerve injury models of neuropathic pain in the rat and pain was promoted by the induction of the complement cascade through C5a (Griffin et al., 2007). The role of complement in nervous system diseases can be both harmful and beneficial (reviewed in (Rus et al., 2006)). Blocking C1q was shown to be beneficial for ischemic neurons (Huang et al., 1999), and in the case of VRA, our results suggest a neurodegeneration-promoting effect of C1q. C1q has also been reported to have a role in the clearance of apoptotic cells (Nauta et al., 2002) and the observed correlation with neurodegeneration could thus also merely be a consequence of the degree of cell death.

5.4.3 The Affymetrix methodology

The Affymetrix microarrays consist of chips with *in situ* synthesized oligonucleotides to which sample cRNA is hybridized. The RG-U34A microarrays used in this study represent approximately 7000 full-length sequences and 1000 EST clusters (http://www.affymetrix.com). In contrast to cDNA microarrays, each target on Affymetrix arrays is represented by a probeset including both match- and mis-match probes. The signal detected from each target is thus calculated based on several independent hybridizing sequences and irrelevant binding is subtracted. An important difference to cDNA microarrays is that only one sample is hybridized to each chip, making comparisons possible between all chips in an experiment, in the case of three replicates this allows for nine comparisons instead of only three. Considering the wealth of data obtained in microarray analyses, stringent statistics and filtering criteria are needed to lower the false positive rate. According to Lockhart and Winzeler, the false positive rate can be as low as 1/10 000 by analyzing independent replicates (Lockhart and Winzeler, 2000). We employed an analysis based on the Affymetrix Microarray Suite software for generating detection, signal, and mean signal log ratios (MSLR). Identification of regulated genes was based on specific filtering criteria (transcript present in 2/3 of reference chips, “change” call in 7/9 chip comparisons and a MSLR >0.5 or <0.5). There are numerous ways of handling and analyzing microarray data, and each method will generate its own set of regulated genes. Additional analysis methods could serve as tools to identify robustly regulated genes in the dataset by comparing the overlap between our obtained results with those of alternative analyses.

6 GENERAL DISCUSSION

The studies included in this thesis aim at characterizing the genetically dependent differences in the outcome after experimental nerve injury and to map these to distinct genetic regions or genes that can be tested for impact on experimental phenotypes and for association to human disease. Identifying new phenotypes or QTLs regulating specific phenotypes is thus merely the start point for the goal of manipulating human disease. A discussion on the current status of follow-up projects and possible applications of obtained results will follow here.

6.1 CANDIDATE GENE IDENTIFICATION

For *Vra4*, the candidate gene *Mhc2ta* was identified after fine-mapping in AILs and could be tested for association to human disease (Paper II). The gene effect on experimental disease was later confirmed in *Vra4*-congenic strains (Paper III). The strong phenotypic effect of *Vra4* was probably responsible for allowing gene identification at an early stage. For QTLs with lower effect, information from congenic strains may be necessary for narrowing the QTL and to allow candidate gene identification. Such congenic mapping is performed by following the phenotypic effect in overlapping intra-QTL recombinant congenic strains in order to identify the minimal shared fragment conferring e.g. protection from disease. Candidate gene identification in congenic strains can be made possible by characterizing sub-phenotypes that may identify involved regulatory pathways. To obtain single-gene congenic rats with retained phenotype is the ultimate positional cloning of a candidate gene, but is hampered by the lack of polymorphic markers and the position-specific recombination events needed. Therefore, complementary strategies need to be followed e.g. creation of haplotype maps, gene and protein expression analyses, gene sequencing and functional assays.

6.1.1 Current status of *Vra1-3*

Characterization of *Vra1-3* is ongoing. To date, *Vra1* and *Vra2* have been confirmed as linked to neurodegeneration, but linkage of CD3+ cells to *Vra2* and *Vra3* has not been confirmed. For the neurodegeneration phenotype, *Vra1* has been most extensively studied and show reproduced linkage in AIL G8 and G10 as well as in congenic strains (Figure 12 and 13). PVG^{av1} alleles at the *Vra1* locus are thus protective and are associated with less motoneuron loss after VRA in DA.PVG^{av1}-*Vra1* congenic strains. The fine-mapping of *Vra1* has followed the pattern of identification in F2, QTL narrowing in AILs and confirmation and congenic mapping in congenic strains. So far, no candidate genes have been identified for this region.

Only males were included in the AIL experiments due to a higher inter-individual variation in the neurodegeneration phenotype observed in females of the F2 generation. In total, 126 males from the G8 and 186 males from the G10 generation were included. In both AIL generations, the allele frequencies at the *Vra1* max marker D8Rat138 were

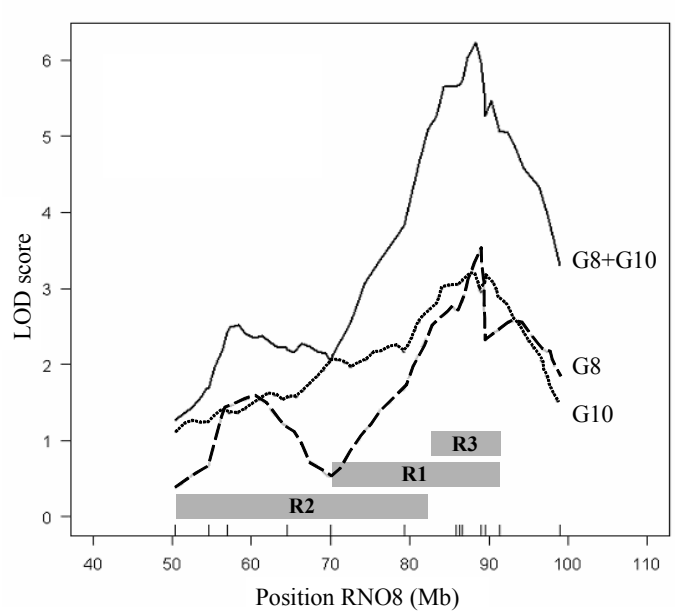

Figure 12. Confirmation of *Vra1* in AIL G8 (dashed line), G10 (dotted line) and combined (solid line) linkage analyses. The max marker LOD score for the combined analysis is 6.0 and the LOD support interval of 1.5 corresponds to 19.6 Mb. Positions of the congenic fragments in DA.PVG^{av1}-*Vra1* recombinant R1, R2 and R3 strains are shown in relation to the QTLs.

skewed towards excess of DA alleles, with a frequency of 0.62 compared to 0.38 for PVG^{av1} alleles. When looking at the genotypes, the frequencies of heterozygotes were 0.52 and 0.49 in G8 and G10, respectively. The excess of DA alleles was thus reflected by the homozygotes: 0.36 DA/DA and 0.12 PVG^{av1}/PVG^{av1} in G8 and 0.38 DA/DA and 0.13 PVG^{av1}/PVG^{av1} in G10. In the F2 generation, we observed no skewed genotypes at *Vra1*. The genotype pattern in the AIL probably reduced the power to detect linkage to protective PVG^{av1} alleles, and could explain the relatively low LOD scores in the individual AIL experiments (2.5-3.5) but the increased LOD score (6.0) in a combined analysis of the two AIL generations. The combined analysis was performed in R/QTL with generation as a covariate and resulted in a narrowed CI (19.6 Mb) compared to the F2 CI (55.4 Mb). The max marker (D8Rat138) was the same in G8, G10 and in the combined analysis (Figure 12).

Several overlapping congenic rat strains have been bred for *Vra1*. The size and position of the congenic fragments in the three strains tested so far; DA.PVG^{av1}-*Vra1*-R1 (R1), DA.PVG^{av1}-*Vra1*-R2 (R2) and DA.PVG^{av1}-*Vra1*-R3 (R3) are illustrated in Figure 12. A protective effect of *Vra1*, i.e. decreased motoneuron loss after VRA, has been confirmed in R1 and R3 and mapped to a 9.1 Mb region harbored by the R3 congenic strain (Figure 13). This rather small fragment can be compared with the 55 Mb CI in the F2 and the 19.6 Mb CI obtained in the combined AIL analysis (Table 3). Breeding of smaller recombinant congenic strains is ongoing to further reduce the size of *Vra1*. Reciprocal congenic strains are also bred to investigate if DA *Vra1*-alleles could mediate increased susceptibility to motoneuron loss when introgressed on the PVG^{av1} background.

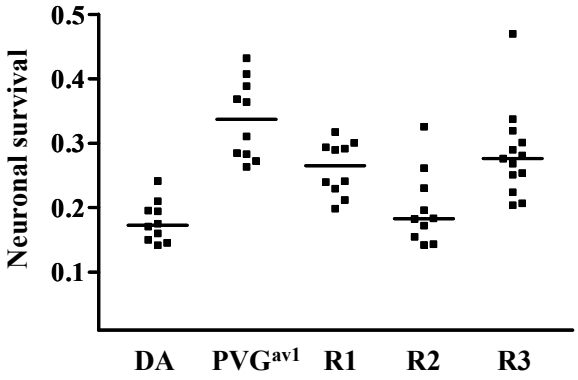

Figure 13. Neuronal survival in *Vra1*-congenic strains. DA.PVG^{av1}-*Vra1*-R1, R2 and R3 are overlapping recombinants in the region. Significant higher neuronal survival 21 days after VRA compared to DA was seen in the R1 ($p<0.05$) and R3 ($p<0.01$) congenic strains, Kruskall-Wallis and Dunn's post test.

Very few QTLs regulating neuronal sensitivity to degeneration have been reported. A locus on mouse chromosome 1 that regulated sensitivity to MPTP induced neuron loss in substantia nigra pars compacta was identified in an intercross between C57/BL/6J and Swiss-Webster mice (Cook et al., 2003), and an intercross between BALB/cByJ and C57BL/6J mice identified a locus on mouse chromosome 6 that mediated protection to age-related retinal degeneration (Danciger et al., 2003). Neither of these QTLs overlaps with *Vra1*. The reproduced linkage of *Vra1* to neurodegeneration may thus give new insight to the processes and genetic factors determining neuronal susceptibility.

Vra2, with suggestive linkage to neurodegeneration and significant linkage to CD3+ cells in the F2 has given inconsistent results for both phenotypes when analyzed in the AIL. The fact that this locus was originally linked to both phenotypes and the dual roles of T cells in neurodegeneration and neuroprotection makes a combined analysis interesting. Characterization of *Vra2* is currently being pursued by further AIL analyses and congenic breeding.

Table 3. Summary of the fine-mapping of the *Vra1* locus linked to neurodegeneration in DAxPVG F2 intercross, DAxPVG^{av1} AILs and congenic strains. Positions are given for markers flanking a LOD support interval of 1.5 for F2 and AIL, and for the minimal congenic fragment with retained phenotypic effect.

	Flanking markers	Position (RNO:Mb)	Size (Mb)	Max marker	LOD
F2	D8Rat99-D8Rat129	8: 43.56 - 98.95	55.4	D8Rat205	5.5
AIL G8+G10	D8Rat26-D8Rat129	8: 79.35 - 98.95	19.6	D8Rat138	6.0
Congenics	D8Rat24-D8Got145	8: 82.25 - 91.33	9.1		

In conclusion, the whole genome scan of VRA responses in Paper I identified four QTLs that are now at different stages of evaluation. *Vra4* has come as far as identifying a candidate gene with reproduced association to human disease and clinical effects in congenic rats. Focus will now be on characterizing the protective effect of *Vra4* in the congenics in order to increase understanding of the impact on human inflammatory disease. Any clinical effect on additional disease models to EAE will be interesting to evaluate, as well as sub phenotypes such as lymphocyte profile and antigen presenting capabilities.

Vra2 and *Vra3* have to be further investigated with regard to linkage to CD3+ cells and neurodegeneration.

Vra1 has been fine mapped by both AIL and congenic strains, which has resulted in a significant reduction of the QTL size. Still, many genes are located in the present 9.1 Mb large region. Thus, further congenic mapping combined with sequencing, expression analysis and functional assays of candidate genes have to be performed in order to identify candidate genes. The possible congenic effect in additional models for neurodegeneration will also be pursued. The fact that *Vra1* indeed mediates neuroprotection may give new insight into both pathogenesis and treatment of the heterogeneous group of neurodegenerative disorders.

6.2 *MHC2TA* ASSOCIATION TO INFLAMMATORY DISEASE

The reported association of *MHC2TA* to three different diseases with inflammatory components in Paper II has been followed by a number of association studies of the -168 A/G (rs3087456) SNP with both positive and negative findings (Table 4).

6.2.1 MS and RA

A German study did not find any association of the *MHC2TA* -168 A/G (rs3087456) SNP to MS, RA, narcolepsy or Wegener granulomatosis (Akkad et al., 2006). Accordingly, no association to RA and MS was found in a Spanish study when using only the -168 A/G SNP. On the contrary, a haplotype analysis with 1614 G/C (rs4774) on the same material showed positive association to both RA and MS (Martinez et al., 2007b). In addition, Mihalova and co-workers have reported association of *MHC2TA* in a UK MS material by a haplotype effect (Mihalova et al., 2007). Genes with moderate impact on disease may be important risk factors for specific sub phenotypes or when in combination with other genetic or environmental risk factors. A recent study reported association of *MHC2TA* 1614 G/C to MS cases with active HHV-6A (Martinez et al., 2007a). Association of the 1614C allele was found for MS-HHV-6A compared to MS ($p=0.0001$), as well as to the control group ($p=0.001$), arguing for a gene-environment interaction in this context.

Three different meta-analyses on US, UK, Japanese and European family materials have shown consistent linkage for RA to 16p12-13, the region harboring *MHC2TA* (Fisher et al., 2003; Choi et al., 2006; John et al., 2006) (Table 5). In one of the studies, conditioning for HLA (DRB1) indicated interactive effects between 16p and HLA

Table 4. Summary of *MHC2TA* association studies.

Author	Outcome	Clinical material	n cases	n controls	Population	Risk (r) / Protective (p) allele	Comment
Mihalova, T 2007	+	MS	474	775	UK	(p) GTTA and GTA	Haplotype effect 3/4 SNPs.*
Martinez, A 2007		MS	77	520	Spain		Association of HHV-6A positive MS both compared to MS and controls.
	+	MS+HHV-6A	22	=	=	(r) rs4774(C)	
O'Doherty, C 2007	(+)	MS	440	316	N Ireland	(p) rs4774(C)	2 SNPs, association also for pooled material.
	(+)	RA	293	=	=	=	
	+	JIA	74	=	=	=	
Martinez, A 2007	(-)	MS	396	519	Spain	(r) rs3087456(G)/rs4774(C)	Haplotype effect. Same tendency for MS as for RA.
	+	RA	350		=	(p) rs3087456(A)/rs4774(C)	
	-	IBD	663		=		
Akkad, DA 2006	-	MS	646	463	Germany		Controls were healthy blood donors.
	-	RA	319	=	=		
	-	JRA	237	=	=		
	-	Narcolepsy	174	=	=		
	-	WG	178	=	=		
Swanberg, M 2005	(+)	MS	548	528	Sweden	(r) rs3087456(G)	Association to MS when all controls used.
	+	RA	1288	709	=	=	
	+	MI	387	387	=	=	
Iikuni, N 2007	+	RA	1128	455	Japan	(r) rs3087456(G)	The risk allele is the most frequent.
Harrison, P 2007	-	RA	733	613	UK		
Yazdani-Biuki, B 2006	-	RA	362	351	Austria		1709 additional controls genotyped.
Newman, WG 2006	-	RA	1187	462	Canada		
Eyre, S 2006	-	RA	1401 (813)	2475 (532)	UK		6 SNPs. Higher n only for rs3087456.
Orozco, G 2006	(+)	RA	748	676	Spain	(r) rs3087456(A)	rs3087456(A) risk allele reported as false positive. Varying allele frequency.
	-		287	287	Argentina	=	
	-		278	478	Sweden	=	
Linga-Reddy, MV 2007	-	SLE	334	478	Swedish		
Koizumi, K 2005	+	SLE	100	100	Japan	(r) 485A/G	8 SNPs.
Lindholm, E 2006	+	Botnia study	4432		Nordic	(r) rs3087456(G)(AG)	Association to cardiovascular mortality and metabolic syndrome.
	+	T2D	3065 (+1557)	2864	Sweden	=	
	+	MI	1222	2345	Sweden	=	
Ghaderi, M 2006	+	AAD	128	406	Italy	(r) rs3087456(G)	Risk independent of HLA.

*MS-multiple sclerosis, HHV-human herpes virus, RA-rheumatoid arthritis, JIA-juvenile idiopathic arthritis, IBD-inflammatory bowel disease, JRA-juvenile RA, WG-Wegener granulomatosis, MI-myocardial infarction, SLE-systemic lupus erythematosus, T2D-type 2 diabetes, AAD-autoimmune Addison´s disease. *rs7501204 (G), rs6498114(T), rs6416647(T), rs3087456(A). =Same as above.*

Table 5. Three meta-analyses of four materials each reported linkage to RA for 16p(13-q12.2), the region harboring the *MHC2TA* gene. ASP-affected sibling pair.

Author	n ASPs	Population	Linkage	Comment
Fisher, SA 2003	636	US, UK, Japan, Europe	16p13-q12.2	
John, S 2006	886	US, UK	16p	Interaction with HLA indicated.
Choi, SJ 2006	964	US, UK, Europe	16p13-q12.2	

(John et al., 2006). Up to date, RA has also been the most extensively studied disease with regard to association to *MHC2TA*. No association of -168 A/G to RA was reported from six studies performed in German (Akkad et al., 2006), Austrian (Yazdani-Biuki et al., 2006), British (Eyre et al., 2006; Harrison et al., 2007), Canadian caucasian (Newman et al., 2006), Argentinian and Swedish (Orozco et al., 2006) populations. In contrast, significant association to RA was found for -168 A/G in a Japanese study (Iikuni et al., 2007), for 1614 G/C in juvenile idiopathic arthritis (JIA) from Northern Ireland (O'Doherty et al., 2007) and in the Spanish study mentioned above when analyzing the haplotype between -168 A/G and 1614 G/C (Martinez et al., 2007b). The study performed in Northern Ireland also included MS and RA cases, and they showed the same tendencies as JIA. In addition, the C allele at 1614 G/C was significantly associated to disease when pooling the three materials (O'Doherty et al., 2007). The C allele of 1614 G/C was the risk-associated allele in the haplotype associated to MS, RA and in HHV-6A-positive MS (Martinez et al., 2007a; Martinez et al., 2007b), but was found to be protective in the three materials from Northern Ireland (O'Doherty et al., 2007).

A meta-analysis of ten of the above-mentioned association studies of *MHC2TA* -168 A/G association to RA showed no association to disease (Bronson et al., 2007). Among the included studies, three had reported positive association.

6.2.2 Other inflammatory diseases

For other autoimmune diseases, two *MHC2TA* association studies on systemic lupus erythematosus (SLE) and one on autoimmune Addison's disease (ADD) have been reported. The first SLE study was conducted in a Japanese population and showed an increased allele frequency of a newly discovered SNP 485 A/G in patients compared to controls (Koizumi et al., 2005). The second study found no association of *MHC2TA* -168 A/G to SLE in a Swedish material (Linga-Reddy et al., 2007). Ghaderi and coworkers found a HLA-independent association of -168 A/G to autoimmune ADD in Italy (p=0,003, OR 1,72) (Ghaderi et al., 2006).

The most extensive study published so far reported association of *MHC2TA* -168 A/G to cardiovascular mortality after MI and to the metabolic syndrome (Lindholm et al., 2006). This study included a total of 11064 individuals from three different study populations; the Botnia type 2 diabetes study, type 2 diabetes patients, MI cases and controls. Of note, there was no association to MI alone but to cardiovascular mortality after MI.

6.2.3 Conflicting results

Any clear conclusion on the impact on disease from the *MHC2TA* gene is hard to draw based on these conflicting results. Important points to consider when interpreting the reports on associations are the SNPs used, the ethnicity of the studied population, the sample size and the use of suitable controls.

Of the reported studies, many were restricted to the -168 A/G SNP located in the *MHC2TA* type III promoter. Among the studies that analyzed SNPs additional to -168 A/G, only one found no association. This study by Eyre and coworkers analyzed five additional SNPs in a subset of the studied cohort, all located in the promoter region upstream the -168 A/G SNP (Eyre et al., 2006). It would be very interesting to include SNPs in the coding and intronic sequences of *MHC2TA* in this extensive material to be able to elucidate the haplotype patterns and their possible effect on association. In our study, haplotype analysis including 1614 G/C did not improve association (Paper II), but unless the -168A/G polymorphism is the functional variant, these results may reflect genetic heterogeneity between the studied populations affecting the distribution and size of haplotypes. The optimal set of markers has to be evaluated for each population studied. Sample size is also crucial when studying the genetics of complex disease. It can, however, be argued that by defining distinct sub-phenotypes or disease entities, smaller sample size can be informative (Martinez et al., 2007a).

Significant differences in *MHC2TA* -168 A/G allele frequencies are obvious in the different studied populations (Swanberg et al., 2005; Lindholm et al., 2006; Orozco et al., 2006; Iikuni et al., 2007; Linga-Reddy et al., 2007). Among the three studies performed in the Swedish population, two show very similar AG+GG genotype frequencies in population based controls, 36% in Paper II compared to 37% in the study by Lindholm and coworkers (Lindholm et al., 2006). Both these studies showed association of *MHC2TA* to disease. The third study had a significantly higher AG+GG genotype frequency of 45% in the controls and found no association (Linga-Reddy et al., 2007). The reason for this variation in genotype frequency could not be explained by the latter study, but could not be attributed the population substructure.

6.3 COMBINING LINKAGE AND EXPRESSION

The microarray expression study presented in this thesis (Paper IV) was conducted on DA and PVG parental strains. The data can thus be used as a source for new phenotypes differing between the strains and to search for co regulated genes and networks involved in the response to nerve injury. To perform expression QTL (eQTL) mapping, genetic heterogeneous animals must be studied, e.g. an F2 population. In addition, expression profiling on congenic strains with a well defined introgressed fragment would open possibilities for comparisons with the data obtained from parental strains and give insight into which genes, pathways and processes are affected by the genetic transfer.

Comparison of physiological QTLs (pQTLs) with expression data has successfully identified candidate genes for defective metabolism and insulin resistance in the hypertensive rat (Aitman et al., 1999; Collison et al., 2000), airway hyper responsiveness in the mouse (Karp et al., 2000), diabetes (Eaves et al., 2002) and complex drug-related behaviors (Tabakoff et al., 2003). The expression data obtained in Paper IV from DA and PVG parental strains can be used in a similar way to complement pQTL data from the F2 intercross in Paper I. So far, we have not been able to successfully couple these studies. The candidate gene *Mhc2ta* in *Vra4* has such a strong effect that it is likely to have been identified in a combined analysis, but the microarrays used lacked the probe sequence for this gene. We could, however, see the trans-effect of the cis-acting polymorphisms in *Mhc2ta* reflected in the expression differences of *Cd74*. The reason to the current lack of success in identifying overlapping p- and e-QTLs in our data sets may result from false positive pQTLs, false negative expression differences, or trans-acting eQTLs that will result in observed expression differences that are more difficult to identify as overlapping e- and pQTLs.

If we had an early marker for subsequent neurodegeneration, a whole genome scan could be performed during the early injury response and expression profiles be correlated to the proneness for neurodegeneration. eQTLs identified in such an analysis would be extremely interesting in a neuroprotective point of view. Ideally, one could perform eQTL mapping on a DAxPVG^{av1} F2 population or recombinant inbred (RI) lines.

Mapping of eQTLs in a heterogeneous F2 population would have the advantage of informative genotypes at each locus, but the disadvantage of having just one replicate sample for each genetic setup. A recent obesity associated eQTL mapping in rodents was performed in a F2 mouse intercross (Schadt et al., 2003). In the case of VRA, RNA would have to be amplified in order to get enough cRNA from one individual for chip hybridization. An alternative is the use of RI lines, where animals from the F2 generation are inbred to create new inbred strains (Broman, 2005). RI lines will have a unique assortment of parental alleles at each locus, but be homozygous across their genome. As for any inbred strain, homozygozity allows for replicate samples, but the comparison of several RI lines combines this feature with the possibilities of intercross populations to perform genetic mapping. The resolution of QTL mapping in RI lines is higher compared to intercrosses due to the accumulated recombinations during many generations of inbreeding. A limitation is the number of RI lines available. An eQTL

analysis of 30 RI lines originating from the spontaneously hypertensive rat and the BN strain mapped cis- and trans-acting eQTLs for thousands of transcripts (Hubner et al., 2005). Many cis-acting eQTLs have large effects and behave as monogenic traits, while trans-acting eQTLs can regulate multiple transcripts and each regulated transcript can be under the control of multiple trans-acting eQTLs.

An important factor to consider when performing eQTL analysis is that false cis-acting eQTLs may be detected due to sequence variation in the transcript region targeted by the microarray. The resulting mis-match to probes on the array will lead to altered hybridization and interpreted as a change in expression. For the Affymetrix arrays, a method that identifies deviating probes in a probe set and removes them from the analysis was recently developed (Alberts et al., 2007). Confirming identified eQTLs by RT-PCR is another way to detect allele-dependent false positive cis-acting eQTLs.

Without any limitations in resources, the data preceding and the data included in this thesis suggest development of RI lines between strains characterized with regard to VRA responses, i.e. DA, BN, Lew, PVG, ACI and E3. For unique alleles present in only one of the founders, 576 of these theoretical 6-way RI lines would give 16 lines with any two locus combination. For biallelic loci, it would be possible to study multiple loci interaction. This would be a very challenging task that could profit from the findings presented in this thesis.

7 CONCLUSIONS

The main conclusion of this thesis is that neurodegeneration and inflammation after mechanical nerve injury in the rat are genetically controlled, can be dissected by standard breeding strategies and that identified candidate genes may be of importance for human disease.

- Nerve injury-induced neurodegeneration and inflammation are controlled by both independent (*Vra1*, *3*, *4*) and shared (*Vra2*) QTLs.

- MHC class II expression in the CNS is controlled by polymorphisms in the *Mhc2ta* gene located in *Vra4*.

- The phenotypic effect of *Vra1* and *Vra4* is retained in congenic rat strains after VRA. In addition, *Vra4* congenic rats are protected from EAE.

- Polymorphisms in *MHC2TA*, the human orthologue to the *Vra4* candidate gene, are associated with inflammatory human disease

- DA and PVG inbred rat strains differ significantly in expression profile after nerve injury, with a pronounced pro-inflammatory phenotype in the DA rat.

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