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GENETIC DISSECTION OF MODELS FOR NEUROINFLAMMATION AND NEURODEGENERATION

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As an adolescent I aspired to lasting fame, I craved factual certainty, and I thirsted for a meaningful vision of human life - so I became a scientist. This is like becoming an archbishop so you can meet girls. – M. Cartmill

ABSTRACT

Many central nervous system (CNS) diseases are characterized by inflammation and nerve cell loss, but the exact relationship between these phenomena is not known. The complex etiology of CNS disorders includes a genetic component. MHC class II molecules are key mediators of immune activation and variations in MHC class II genes are the main genetic determinant of several complex autoimmune disorders. Gene regions regulating complex phenotypes can be mapped in experimental animal crosses. In this thesis, the genetic regulation of the response to mechanical nerve injury and experimental autoimmune encephalomyelitis (EAE) was investigated in populations of intercrossed mice and rats.

The effect of a previously identified gene region, *Vra4,* on MHC class II expression was characterized in DA.PVG^{1av1}-*Vra4* and PVG^{1av1}DA-*Vra4* congenic rat strains after ventral root avulsion (VRA). *Vra4* contains the class II transactivator gene, *Mhc2ta,* which is a transcription factor for MHC class II. The influence of the same region was tested in EAE. The results show that *Vra4* regulates MHC class II on microglia after VRA, as well as risk and severity of EAE. In addition, IFN-γ inducible class II expression on antigen presenting cells (APCs) is dependent on the *Vra4* region. Similar results were obtained in a study of inbred mouse strains, where differential MHC class II expression was observed in the facial nucleus after axotomy of the facial nerve. Congenic strain experiments and sequencing of *C2ta* strongly indicate that polymorphisms in the regulatory region of the pI promoter are regulating this trait. *Vra4/C2ta* had no effect on expression of microglial markers, co-stimulatory molecules or MHC class I, nor T cell infiltration. Additional genetic influence on MHC class II expression was mapped to chromosomes 1 and 7 in a F2 cross between BN and LEW.1N rats, two strains which share *Mhc2ta* haplotype but display differential MHC class II expression after VRA. Analysis of other inflammatory markers in this cross revealed common regulation of several immune related molecules by the same gene region, which may suggest upstream effects.

Finally, the genetic impact on nerve cell death following VRA by two gene regions previously detected in a F2(DAxPVG^{c}) cross, *Vra1* and *Vra2*, was fine mapped in 2 generations of an advanced intercross line (AIL) between DA and PVG^{1av1} rats, as well as in a panel of *Vra1* congenic strains. The effect of *Vra1* on neurodegeneration was reproduced in both AIL populations. Increased support was given by the congenic strains, where PVG alleles in the *Vra1* region on DA background resulted in significantly reduced neuronal loss. These studies also narrowed down the *Vra1* region from 54 to 9 Mb. *Vra2* displayed suggestive linkage to neurodegeneration only in one AIL cohort, but showed an additive effect on the phenotype together with *Vra1*.

To conclude, these results show that neuroinflammation and neurodegeneration are influenced by genetic factors. Identification of genes and pathways will increase our understanding of the molecular pathways of human complex disease.

LIST OF PUBLICATIONS

I. *Vra4* congenic rats with allelic differences in the class II transactivator gene display altered susceptibility to experimental autoimmune encephalomyelitis. Karin Harnesk*, Maria Swanberg*, Johan Öckinger, Margarita Diez, Olle Lidman, Erik Wallström, Anna Lobell, Tomas Olsson, Fredrik Piehl. Journal of Immunology, 2008 Mar 1;180(5):3289-96.
(*These authors contributed equally.)

II. Differential nerve injury-induced expression of MHC class II in the mouse correlates to genetic variability in the type I promoter of *C2ta*. Karin Harnesk, Maria Swanberg, Margarita Diez, Tomas Olsson, Fredrik Piehl, Olle Lidman. Journal of Neuroimmunology. 2009 Jul 25;212(1-2):44-52.

III. Identification of gene regions regulating inflammatory microglial response in the rat CNS after nerve injury. Margarita Diez, Nada Abdelmagid*, Karin Harnesk*, Mikael Ström, Olle Lidman, Maria Swanberg, Rickard Lindblom, Faiez Al-Nimer, Maja Jagodic, Tomas Olsson, Fredrik Piehl. Journal of Neuroimmunology. 2009 Jul 25;212(1-2):82-92.
(*These authors contributed equally.)

IV. Fine mapping of gene regions regulating neurodegeneration. Maria Swanberg, Karin Harnesk*, Mikael Ström*, Margarita Diez, Olle Lidman, Fredrik Piehl. PLoS One. 2009 Jun 15;4(6):e5906.
(*These authors contributed equally.)

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AIL	Advanced Intercross Line
ALS	Amyotrophic Lateral Sclerosis
APC	Antigen Presenting Cell
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
BBB	Blood-brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BLS	Bare Lymphocyte Syndrome
BN	Brown Norway
C1q	Complement factor 1q
C3	Complement factor 3
CFA	Complete Freund's Adjuvant
ChAT	Choline Acetyltransferase
CI	Confidence Interval
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocyte
DA	Dark Agouti
DC	Dendritic Cell
EAE	Experimental Autoimmune Encephalomyelitis
EAN	Experimental Autoimmune Neuritis
EBNA	Epstein Barr Nuclear Protein
EBV	Epstein Barr Virus
GFAP	Glial Fibrillary Acidic Protein
GWAS	Genome Wide Association Study
HLA	Human Leukocyte Antigen
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
LD	Linkage Disequilibrium
LOD	Logarithm Of Odds
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MHC2TA	Major Histocompatibility Complex Class 2 Transactivator
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NGF	Nerve Growth Factor
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDGF	Platelet Derived Growth Factor
PLP	Phospholipid Protein
PPMS	Primary Progressive MS
PVG	Piebald Virol Glaxo

QTL	Quantitative Trait Loci
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
RRMS	Relapsing/Remitting MS
SNP	Single Nucleotide Polymorphism
SPMS	Secondary Progressive MS
TGF	Transforming Growth Factor
Th	T helper
TNF	Tumor Necrosis Factor
VRA	Ventral Root Avulsion

1 COMPLEX DISEASES

1.1 THE CHALLENGE OF MAPPING COMPLEX DISEASES

If a single gene is both necessary and sufficient for the development of a particular trait or disease, this trait or disease is called monogenic or Mendelian. Monogenic disorders, for example Cystic fibrosis, Huntington's chorea and Sickle cell anemia, are typically very rare in a population. In contrast, complex traits or diseases are caused by several different genetic and environmental factors, and are much more prevalent. In fact, a majority of common human disorders, such as arthritis, hypertension, asthma and most cancers, are complex in nature. The low degree of heritability and the large degree of heterogeneity that characterize these disorders make it difficult to identify genes which influence disease risk and/or severity. Nevertheless, the rate of complex disease gene discovery has accelerated the past three decades, largely thanks to the great advantages in biomolecular technology, such as the development of genetic maps based on microsatellite and single nucleotide polymorphisms (SNP), sequencing of the human and other genomes, the possibility of high throughput genotyping with the polymerase chain reaction (PCR) technique and the development of whole genome genotyping technologies. The rapid evolution of computer technology and the internet has also facilitated research through the widespread availability of various data analysis software programs and large genetic databases. Together, the progress in these fields has enabled identification of a vast number of candidate genes for such diverse diseases as malaria (Jallow et al., 2009), ovarian cancer (Song et al., 2009), Crohn's disease (Raelson et al., 2007) and type I diabetes (Hakonarson et al., 2007).

Although the positioning of complex disease genes is a big leap forward, the challenge remains to elucidate the mechanisms by which these genes regulate disease. An increasingly popular technique is the global gene expression technology, which may provide valuable information about the genetic and molecular basis of complex disorders by combining messenger RNA (mRNA) expression analysis on a chip with genetic linkage analysis (Cookson et al., 2009). For detailed studies of genetic regulation and pathways of disease, studies of disease models in susceptible and resistant animal strains are very useful. Such studies are the basis of this thesis.

1.2 THE MAJORITY OF CNS DISEASES ARE COMPLEX IN NATURE.

Common central nervous system (CNS) disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) and Multiple sclerosis (MS). These disorders are a major cause of disability and personal suffering, as well as high societal costs.

AD, PD and ALS are considered to be primarily neurodegenerative diseases. In AD, cholinergic neurons in the forebrain degenerate, which results in cognitive impairment, progressing memory loss and personality change, while PD is characterized by a gradual loss of dopaminergic neurons in the substantia nigra, leading to motor dysfunction and dementia. ALS is a fatal disease where motoneurons in the spinal cord, brain stem and motor cortex are degraded and the patients suffer increasing paralysis. The cause of death is usually loss of respiratory function.

There are Mendelian forms of all three of these diseases. For instance, 5% of Alzheimer cases are caused by mutations in the Presinilin1 and 2 genes (PSEN1 and PSEN2) or amyloid precursor protein (APP) gene (Rocchi et al., 2003). Similarly, 5-10% of patients with ALS carry single gene mutations leading to the familial form of the disease, such as mutations in the superoxide dismutase gene SOD1 (Robberecht, 2000). However, the vast majority are sporadic, relatively late-onset cases with complex etiology. The main genetic contributor to sporadic AD is the $\varepsilon 4$ allele of the Apolipoprotein E (ApoE) gene, which accounts for roughly 20-30% of cases (Kamboh, 2004), while the one of the more promising candidates for PD is the SNCA gene, encoding the α-synuclein protein (Bras and Singleton, 2009).

It is not known what causes neurons to degenerate, but toxic effects of improper protein cleavage and folding have been demonstrated in AD and PD (Bossy-Wetzel et al., 2004). It is likely that the local microenvironment, ability of resident CNS cells to maintain and restore homeostasis and the inherent vulnerability of nerve cells are important factors. In addition, nerve cell damage and neurodegeneration are often coupled with inflammatory events. In animal models of both AD and PD, increased production of immunological molecules is observed in areas of degenerating nerve cells (Matsuoka et al., 2001; Pattarini et al., 2007). Therefore, neurodegenerative diseases also have an inflammatory component. On the flipside, nerve cell death is also evident in the primarily inflammatory disease MS, where the degree of

neurodegeneration correlates well with permanent functional deficits (Bjartmar et al., 2000; Trapp et al., 1998). This illustrates that neuroinflammation and neurodegeneration are not two separate phenomena, although the exact relationship between inflammatory activation and neuronal loss is not known.

1.3 MULTIPLE SCLEROSIS IS A COMPLEX, INFLAMMATORY DISEASE.

MS is a chronic, complex disease of inflammatory character, where the body's own immune cells attack the myelin sheath which surrounds the axons of neurons and facilitate efficient propagation of the action potential. Alongside inflammatory infiltrates and demyelination, there are signs of axonal degeneration, which contribute to disability (Sanfilipo et al., 2006; Tjoa et al., 2005). The clinical course of MS is divided into 3 main categories. The most common is the relapsing/remitting (RRMS) form, where the patient experiences bouts (relapses) of increased symptoms followed by a period of improvement (remission). After variable periods of time, RRMS patients often enter a secondary progressive disease course (SPMS), characterized by a slow worsening of symptoms. A minority of patients develop primary progressive MS (PPMS), which is more neurodegenerative than neuroinflammatory in nature. In PPMS, there are no discernible bouts but rather a steady progression of the disease. In Europe and North America, MS is the most common cause of neurological disability in young adults, second only to trauma. In total, there are roughly 2 million affected individuals worldwide. The disease is generally more prevalent in certain geographic areas, e.g. Scandinavia, North America, Canada and Australia. Women are affected with RRMS twice as often as men.

In MS, demyelinating lesions are present at numerous sites in the CNS, which probably explains the heterogeneous nature of the clinical symptoms. The degradation of myelin and axonal loss lead to impaired neuronal signaling, which causes physical disabilities such as balance disturbance, muscle weakness, loss of vision, numbness, pain and abnormal fatigue, but also cognitive impairment. The treatment options available for MS today are mainly immunomodulatory and aim to lessen the symptoms during relapses. However, the possibility to inhibit progression of disability is limited, and the neurodegenerative process is unresponsive to anti-inflammatory treatment (Buttmann and Rieckmann, 2007). Natalizumab, a drug which prevents

entry of T cells to the CNS by blocking the integrin molecule VLA4, showed promising results, but the use of this treatment is conservative since it has caused opportunistic infections in persons taking other forms of immunomodulatory drugs (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005). New anti-inflammatory treatments are now in various stages of clinical trial, and will hopefully be useful in the management of RRMS.

1.4 THE HLA LOCUS IS THE MAIN GENETIC DETERMINANT OF MS RISK.

In spite of large research efforts, the exact cause of MS remains elusive, but there is solid evidence of a genetic component in the etiology of the disease. In monozygotic twins, the concordance rate is roughly 25%, and the risk of developing MS is higher in first-degree relatives of affected individuals. In contrast, adoptees have the same disease risk as the general population (Compston, 1997; Ebers et al., 1995).

Any genetic variant which regulates the risk and/or severity of a complex disease is likely to exert a modest effect, and may not be present in all affected individuals. Therefore, the identification of such genetic factors is difficult. The first gene region which was shown to influence MS was the Human Leukocyte Antigen (HLA) complex (Jersild et al., 1973). This region remains the major genetic determinant of MS risk, and also regulates the risk of other diseases of inflammatory character, such as Type I diabetes and Rheumatoid arthritis (RA) (Thomson et al., 2007; Thomson et al., 1999). The HLA complex is highly polymorphic, comprises some 4 mega base pairs (Mb) and several immune related genes, including those encoding major histocompatibility complex (MHC) class I and II (the function and regulation of MHC class II are discussed in more detail below). Fine mapping of the HLA region has identified the DRB1*1501 and DQB1*0602 alleles of the MHC class II genes within the HLA locus as the main risk factors for disease (Hillert, 1994; Lincoln et al., 2005). Recent studies on the HLA influence on MS reveal additional levels of complexity, with interactions between MHC class II loci, as well as independent effects of MHC class I genes, in the regulation of MS susceptibility (Brynedal et al., 2007; Dyment et al., 2005; Lincoln et al., 2009).

In later years, non-HLA genes which affect multiple sclerosis have been identified, most of which are involved in the immune system. The strongest candidates include

the interleukin (IL) 2 and IL7 receptors and CLEC16A (Hafler et al., 2007; Lundmark et al., 2007). The IL2 receptor (CD25) mediates clonal expansion of regulatory T cells (Letourneau et al., 2009), which have anti-inflammatory effects, while IL7R regulates T cell survival (Jaleco et al., 2003). Less is known about CLEC16A, but it shows similarities with C type lectins, a family of receptors expressed on immune cells. The fact that these genes are also implicated in other diseases with inflammatory elements e.g. Type I diabetes (Lowe et al., 2007) and inflammatory bowel diseases (Marquez et al., 2009), suggests a common regulation of these types of disorders.

1.5 ENVIRONMENTAL FACTORS AFFECT THE RISK OF MS.

In addition to genetic studies, efforts have been made to identify possible environmental factors that can cause MS, including infectious agents, smoking, dietary factors and climate (Ascherio and Munger, 2007a; Ascherio and Munger, 2007b).

The relationship between Epstein Barr Virus (EBV) infection and multiple sclerosis has been of much interest, and this virus is indeed emerging as a strong environmental component in the etiology of MS. Serafini and co-workers saw a high proportion of EBV infected B cells in inflammatory infiltrates of MS brains (Serafini et al., 2007), and MS patients have been shown to have higher reactivity against Epstein Barr nuclear antigen (EBNA1) (Sundstrom et al., 2008). In addition, EBV specific T cells have been detected in peripheral blood of MS patients (Jilek et al., 2008). There is however no definitive mechanism for how Epstein Barr infection leads to disease.

Smoking increases the risk of MS, and also worsens the prognosis (Hernan et al., 2005; Riise et al., 2003; Sundstrom and Nystrom, 2008), which is interesting in light of a study by Klareskog and colleagues, which shows increased risk for rheumatoid arthritis (RA) in smokers who carry the HLA-DR allele (Klareskog et al., 2006). Klareskog's article also suggests interplay, or combined effects, between genetic and environmental risk factors.

Vitamin D deficiency has been proposed as a risk factor for MS, based on the increased prevalence of the disease in countries with fewer hours of sunlight. Some correlation between serum levels and intake of vitamin D and MS incidence has been observed (Munger et al., 2006). Vitamin D has been shown to have down-regulating effects on the immune response, which argues for a protective effect of the vitamin (Adorini and Penna, 2009).

1.6 EAE IS AN ANIMAL MODEL OF MS.

Experimental autoimmune encephalomyelitis (EAE) is an animal model used to study MS. It can be used in variety of experimental animals, such as primates, guinea pigs, mice and rats. Disease is induced by immunization of the animal with myelin antigens. Antigens include spinal cord homogenate, myelin oligodendrocyte glycoprotein (MOG), phospholipid protein (PLP), myelin basic protein (MBP) or peptides of these proteins. The antigens are mixed with an adjuvant, in some models also with mycobacteria (Complete Freund's adjuvant, CFA), to promote immune response. It is also possible to induce disease by transferring autoreactive T cells from a sensitized to a naïve individual (Bernard et al., 1976; Wenk et al., 1967). Different immunization protocols give rise to different disease symptoms and histopathological characteristics. Thus, immunization of LEW rats with MBP in CFA leads to an acute monophasic disease, characterized by a strong inflammatory activation and little demyelination (Swanborg, 2001). In contrast, MOG gives a relapsing-remitting disease course in Dark agouti (DA) and LEW^{1av1} rats, with both demyelination and inflammation evident in the CNS (Storch et al., 1998). General symptoms of EAE are progressive paralysis, beginning at the tail and moving to hind and front legs, concomitant with weight loss.

The wide use of EAE in MS research has been criticized, on the basis that the animal model resembles acute demyelinating states, such as acute optic neuritis, rather than a chronic, lifelong disease, and because all attempts – with the exception of natalizumab – to develop MS treatments from EAE experiments have failed (Sriram and Steiner, 2005; Steinman and Zamvil, 2005). Nonetheless, EAE shares many characteristics with MS. Myelin sheaths are destroyed and there are signs of inflammatory infiltrates, along with autoreactive T and B cells (Baxter, 2007; Weissert et al., 2001). In addition, EAE susceptibility is influenced by both genetic factors, as is evident from disease inductions in different inbred rodent strains, and environmental factors. Thus, the DA rat is the classical susceptible strain, while the Piebald virol glaxo (PVG) strain is highly resistant. The season of immunization also affects EAE; the risk of developing the disease was higher during summer than winter months in a study

performed in mice by Teuscher et al, which point to environmental regulation of EAE (Teuscher et al., 2004).

To date, some 50 disease regulating gene regions have been identified, most of which contain genes involved in different aspects of the immune system (Olsson et al., 2006). As is the case in MS, the major contributor is the MHC region (Gunther et al., 1978; Moore et al., 1980; Weissert et al., 1998). Non-MHC regions have also been implicated in EAE, for example *Eae18* which is located on rat chromosome 10 and harbors several chemokine genes. The locus influences disease incidence, onset, severity and duration (Jagodic et al., 2004). Other EAE regions have been mapped on rat chromosomes 4 and 10 (Roth et al., 1999), 9 (Dahlman et al., 1999) and 15 (Sheng et al., 2005). In the mouse, regions regulating weight loss and paralysis have been discovered on chromosomes 2, 3 and 8 (Encinas et al., 2001), as well as several loci on chromosomes 2, 5, 6, 11 and 16 which influence incidence and lymphocyte subsets (Karlsson et al., 2003). There is an overlap between EAE regulating regions and gene regions involved in other autoimmune disease models, for example experimental RA (Becanovic et al., 2006; Bergsteinsdottir et al., 2000; Dahlman et al., 1998) and experimental autoimmune neuritis (EAN) (Huberle et al., 2009) which again indicates common pathways for several autoimmune diseases.

2 IMMUNITY AND INFLAMMATION IN THE CNS

2.1 MHC CLASS II MOLECULES ARE KEY COMPONENTS OF THE IMMUNE SYSTEM.

The purpose of the immune system is to protect us from harmful agents in our surroundings, and can be divided into two parts: the innate and the adaptive immunity. The innate immune system provides a rapid, but unspecific protection against invading pathogens. Macrophages, natural killer (NK) cells and neutrophils are all part of the innate immunity, and produce complement proteins as well as destroy infecting bacteria or viruses. They also produce a large array of cytokines and chemokines, which aid in shaping the adaptive immune response by promoting activation, proliferation and migration of adaptive immune cells.

The adaptive immune system is made up by lymphocytes (T and B cells), which are highly specific for the infectious agent (antigen). Because the T and B cells need to undergo activation and clonal expansion to launch an adequate response, the adaptive immunity develops later. T cells are divided in different classes: CD4 T cells, or T helper (Th) cells, and CD8 T cells, also called cytotoxic T lymphocytes (CTLs). Th cells are further categorized into Th1 cells, which promote macrophage activation and CTL function, and Th2 cells, which induce B cell activation and antibody production.

In order for CD4 T cells to become activated and express antigen specific receptors, they need to be presented to the antigen in question. This requires the expression of MHC class II molecules and co-stimulatory molecules by professional antigen presenting cells (APCs), which include macrophages, dendritic cells (DC) and B cells. In the APC, antigens are processed and transported to the surface to be presented on MHC class II molecules. Upon binding the MHC class II:peptide complex and co-stimulatory molecules, CD4 T cells become activated and exert their downstream effects. Moreover, MHC class II is a key player in the development of immune tolerance in the thymus, a process where T cells which recognize self antigens but fail to recognize MHC class II, are eliminated to a large extent. MHC class II is also involved in maintaining an active immune response. Failure to express MHC class II results in a severe, in most cases fatal, immunodeficiency disorder called Bare lymphocyte syndrome (BLS), where functional CD4 T cells are absent (Krawczyk and Reith, 2006).

This illustrates the crucial role played by MHC class II molecules in the immune system.

2.2 CIITA IS A REGULATOR OF MHC CLASS II.

One element involved in the expression of MHC class II is the CIITA protein, encoded by the Major Histocompatibility Class 2 transactivator (MHC2TA) gene (*Mhc2ta* in rat, *C2ta* in mouse). CIITA is a transcription factor which induces expression of MHC class II not by binding the DNA itself, but by coordinating other proteins involved in MHC class II transcription, such as RFX and NF proteins and CREB (see figure 1) (Ting and Trowsdale, 2002). Mutations in RFX or CIITA result in BLS (Reith and Mach, 2001).

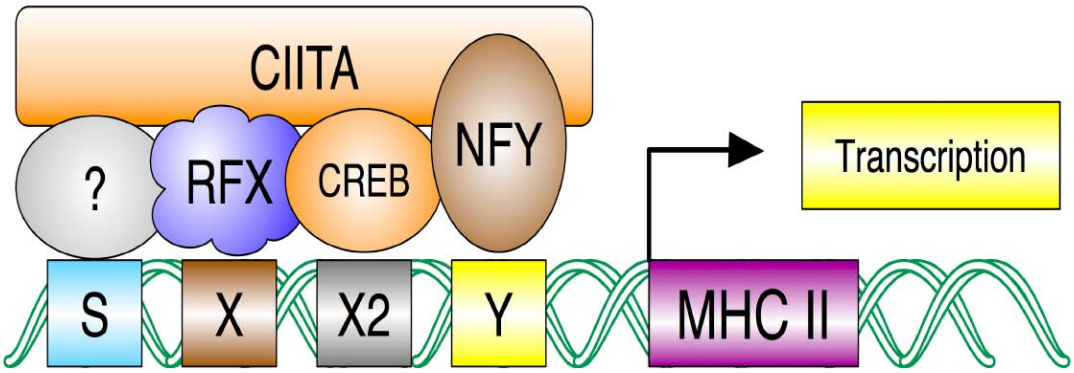

Figure 1. CIITA induces MHC class II expression by coordinating assembly of other transcription factors. Adapted from Reith *et al*, Nature Reviews Immunology 2005.

The MHC2TA gene itself is regulated by several promoters, each giving rise to different isoforms of the CIITA protein. In rat and mouse, there are 3 *C2ta* isoforms, pI, pIII and pIV (see figure 2). These are all present also in humans, with the addition of pII, whose function is yet unknown (Reith et al., 2005).

The different isoforms are expressed in a tissue specific manner, pointing to a complex, cell specific pattern of MHC class II expression. pI is driving MHC class II production in cells of myeloid lineage, i.e. myeloid DC and macrophages, while pIII promotes expression in plasmacytoid DC, T and B cells (LeibundGut-Landmann et al., 2004). pIV is induced in a variety of cell types as a result of interferon (IFN)-γ

stimulation, and is also important for MHC class II expression in thymic epithelial cells (Dong et al., 1999; Piskurich et al., 2006; Suter et al., 2000). In mice lacking the pIV promoter, MHC class II expression was still present in microglia (Waldburger et al., 2001). These data, along with the observations that pI is a myeloid specific promoter, suggest that pI is the main determinant of MHC class II levels in microglia.

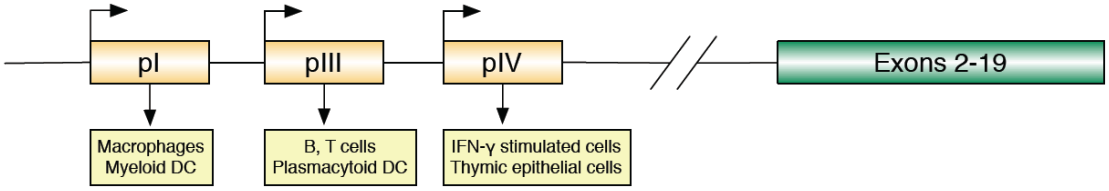

Figure 2. The isoform specific promoters of MHC2TA. The first exon is unique for each isoform. Adapted from Reith *et al*, Nature Reviews Immunology 2005.

The role of MHC2TA in human disease is not fully understood. Swanberg et al found an association of a SNP in pIII to MS, RA and myocardial infarction (Swanberg et al., 2005). Since the publication of Swanberg's study, several groups have tried to replicate the results with limited success (Akkad et al., 2006; Harrison et al., 2007; Yazdani-Biuki et al., 2006). The negative results may be explained by low degree of power, and/or ethnic variations in the populations studied. A large study by Lindholm and co-workers revealed an association of MHC2TA to mortality following myocardial infarction (Lindholm et al., 2006). In addition, MHC2TA has been associated to RA in a Spanish population (Martinez et al., 2009), and to autoimmune adrenal insufficiency in Italian patients (Ghaderi et al., 2006). Interaction between MHC2TA polymorphisms and Human herpes virus (HHV) 6 infection in the etiology of MS was suggested by (Martinez et al., 2007), but these results have not been repeated. It is clear that the precise impact of MHC2TA, and in essence quantitative levels of MHC class II in different cell types, needs to be elucidated further.

2.3 THE CNS IS CAPABLE OF ELICITING IMMUNE RESPONSES.

Previously, the CNS was thought to be protected from immune activation, or "immune privileged". It was believed that resident brain cells were unable to express MHC class

II, that the blood-brain barrier (BBB) was prevented entry of lymphocytes, cytokines and other inflammatory agents and that CNS antigens could not be presented to T cells in the periphery. Although brain immunity differs somewhat to the peripheral variety, the notion of CNS immune privilege as absolute has had to be rejected. Instead, the CNS should be viewed as a *conditionally* privileged organ. Activated lymphocytes are capable of crossing the BBB, and immunological processes do take place near the meninges, circumventrical organs, and ventricles (Galea et al., 2007). In addition, the brain responds to systemic infection and inflammation by increased activation of the hypothalamic-pituitary adrenal axis and behavior modification – so-called sickness behavior, e.g. lethargy, loss of appetite, and tiredness (Dantzer, 2004; Dantzer et al., 2008). Finally, resident CNS cells, i.e. neurons and glia, are capable of promoting and regulating local immune processes as well as the permeability of the BBB. There are three main classes of glia in the brain; astrocytes, microglia and oligodendrocytes, with the first two being known as immunological players. In summary, these data show that complex neuroimmune interactions take place, and that the CNS itself has the capacity to induce immune responses.

Microglia are of mesodermal origin, and are a subclass of mononuclear phagocytes (Giulian et al., 1995). They remain in a resting state in the intact CNS, but monitor the microenvironment of the brain and rapidly react to any irregularities, such as trauma or infection (Kreutzberg, 1996; Raivich, 2005). When activated, microglia proliferate and undergo morphological and molecular changes, demonstrating phenotypes which are of importance for macrophage function: They express MHC class II and co-stimulatory molecules, suggesting antigen presenting and T cell activation properties (Gehrmann et al., 1993; Hickey and Kimura, 1988), in addition to complement factor receptors (Graeber et al., 1988; Roy et al., 2006), pattern recognition receptors (PRRs) (Olson and Miller, 2004), and cytokine receptors (Wang et al., 2006). Microglia also produce a wide range of cytokines, including IL-1b, IL-12, TNF-α (Aravalli et al., 2005; Yan et al., 2001), IL-18 (Hedtjarn et al., 2002) and IL-10 (Gonzalez et al., 2009), as well as reactive oxygen species (ROS) (Colton and Gilbert, 1987). Along with the phagocytic capacities of microglia, these features have rendered them the name “macrophages of the brain”.

In the healthy brain, **astrocytes** are essential for ion homeostasis and maintenance of an appropriate chemical milieu for neurotransmission. A key function of astrocytes is to mediate uptake of the neurotransmitter glutamate, which prevents damage to neurons by excitotoxicity and modulates neuronal signaling. Astrocytes also regulate the integrity of the BBB, by secretion of various cytokines. IL-1, IL-6, IL-10 and TNF-α increase the permeability of the BBB, while TGF-β tightens it, as reviewed by (Nair et al., 2008). Thus, astrocytes influence the degree of infiltration of peripheral immune components into the brain. In addition, astrocytes express chemokines, which suggests a role in the recruitment of immune cells to the site of inflammation (Calderon et al., 2006; Kalkonde et al., 2007).

The antigen presenting capacity of astrocytes has been debated. Although they do express MHC class II *in vitro* (Soos et al., 1998), the role of astrocytes as functional APCs during disease has not been determined (Aloisi et al., 2000; Shrikant and Benveniste, 1996; Stuve et al., 2002).

Neurons are capable of expressing MHC class I (Neumann et al., 1995) and have been seen to inhibit MHC class II expression on astrocytes and microglia, indicating a regulatory role of neural cells (Lee et al., 1992; Neumann, 2001). There is also data which suggest that neurons induce apoptosis in T cells via the Fas-FasL pathway (Flugel et al., 2000). The previous conception that neurons are passive bystanders during inflammatory events has thereby had to be modified.

2.4 INFLAMMATION HAS DUAL ROLES IN THE CNS.

Prolonged inflammation and excessive production of inflammatory agents is commonly believed to have detrimental effects in the central nervous system, and there is indeed ample evidence that this is the case. Inflammation has been implicated in neurodegenerative disorders, such as PD and AD. During inflammation, free radicals and ROS, such as nitric oxide (NO) are produced in large amounts and these molecules have been shown to be harmful to nerve cells and contribute to the loss of dopaminergic neurons in PD (Boje and Arora, 1992; Gao et al., 2008). In addition, long term inhibition of NO synthesis by macrophages and microglia lead to decreased inflammation and demyelination in EAE rats (Danilov et al., 2006). Comparisons between AD patients and healthy controls show higher levels of inflammatory

proteins in microvessels from patients (Grammas and Ovase, 2001), while chemokine expression by resident CNS cells has been implicated in the initiation and disease progression of EAE (Columba-Cabezas et al., 2003; Karpus and Kennedy, 1997); also suggesting that inflammation leads to negative consequences in disease.

However, immune activation is not necessarily detrimental to the CNS. In the periphery, it is well established that inflammation is essential for wound healing, and there is a large wealth of data which indicate a protective and regenerative role of inflammatory cells and proteins also in the CNS. For instance, inhibition of neutrophils impaired outcome and decreased axonal sparing after spinal cord injury in the mouse, suggesting that neutrophils promote recovery and wound healing (Stirling et al., 2009). Studies in knock-out mice have shown that neurodegeneration in Alzheimer models is increased in the absence of complement factor 3 (C3) (Wyss-Coray et al., 2002) and that failure to express the pro-inflammatory cytokine IFN-γ leads to increased proliferation of disease mediating T cells in EAE (Chu et al., 2000).

In addition, inflammation and immune activation often entails an increased production of neurotrophins, which promote neuronal survival. EAE induction in rats subjected to mechanical nerve injury revealed a neuroprotective effect by systemic immunity, likely due to production of neurotrophic factors by CNS infiltrating T cells (Hammarberg et al., 2000a). Similarly, activated T and B cells and peripheral and infiltrating mononuclear cells from MS patients express brain derived neurotrophic factor (BDNF), which may indicate protective effects by immune cells (Gielen et al., 2003; Kerschensteiner et al., 1999). These results suggest a more complex relationship between inflammatory activation and CNS integrity.

The impact of microglia in neurodegeneration and neuroprotection has been investigated at great length, but whether activated microglia aid in exacerbation or remission of disease symptoms is as yet unclear. One might speculate that the state of activation and specific subgroup of this cell type, as well as the surrounding microenvironment, is of importance for the outcome in terms of neurogenesis and nerve cell survival following and during CNS disease.

In a model of epilepsy, inhibition of microglia activation increases the number of newly formed neurons after epileptic episodes (Ekdahl et al., 2003), and *in vitro* studies indicate that activation of microglia and release of proinflammatory cytokines

promote neurodegeneration (Christov et al., 2004; Gao et al., 2002). The fact that activated microglia express APP, which is a prominent feature of Alzheimer's disease, suggests a role in the promotion of neurodegeneration (Banati et al., 1993). In contrast, induction of innate immunity in the brain, with concomitant microglial activation, results in recruitment of oligodendrocyte progenitors to sites of injury, thereby promoting remyelination and repair (Glezer et al., 2006). Activated microglia are also a source of neurotrophic factors, which may favor nerve cell survival and regeneration after stroke (Wang et al., 1997), spinal cord injury (Dougherty et al., 2000), or during AD (Burbach et al., 2004).

To summarize, the role of neuroimmune activation in pathological states remains unclear, but it is likely that a tightly regulated balance between different immunological molecules and cells is of importance for the consequences of an inflammatory event in terms of nerve cell damage. In order to fully understand the biology of diseases such as MS, AD or PD it is crucial to gain knowledge about the kinetic and functional relationship between CNS inflammation and CNS integrity.

2.5 INFLAMMATION AND CELL DEATH CAN BE STUDIED IN NERVE INJURY MODELS.

In order to study the local inflammatory process in the CNS, it is necessary to use a model which does not compromise the integrity of the BBB, or induce a systemic immune response, since this would make it very difficult to distinguish local inflammatory activity from that originating from infiltrating peripheral cells. Mechanical nerve injury models are ideally suited for this purpose, and are also useful for investigating neuronal loss. A wide variety of nerve injury models, including traumatic injury models and those where peripheral axons are subjected to insult, have been studied over the years. The types of lesions include traumatic brain or spinal cord injury, nerve pinch or crush, axotomy (where the axon is transected peripherally) and nerve avulsion (where the nerve is pulled from the CNS). The response to axonal injury is dependent on several factors, such as the age and sex of the animal and the severity of the lesion. Thus, neonatal animals normally display a more detrimental outcome than do adult individuals, and lesions located distally from

the nerve cell soma are milder in nature. However, there are some general features which can be observed after nerve injury.

Axonal insult results in a retrograde reaction, with an intricate interplay between neurons and glial cells. Around 3 days after injury, motoneurons show signs of chromatolysis, e.g. swelling and dispersal of Nissl bodies (Lieberman, 1971). In addition, injured neurons display an increased production of metabolic and growth factors, e.g. BDNF, nerve growth factor (NGF) and insulin-like growth factors (IGF), and neurotrophin receptors (Hammarberg et al., 2000b; Koliatsos and Price, 1996; Piehl et al., 1994). In contrast, proteins involved in neurotransmission, such as choline acetyltransferase (ChAT) are downregulated (Koliatsos et al., 1994; Penas et al., 2009). A process known as synaptic stripping takes place, where the cell soma is detached from the axon; about 1 week after insult, motoneurons begin to die. TUNEL staining and morphological characterization of nerve cells indicates that the mode of nerve cell death is apoptosis, rather than necrosis (Martin et al., 1999). The delay in neurodegeneration also argues for an apoptotic process, as well as upregulation of proteins involved in apoptosis after nerve axotomy (Baba et al., 1999) and nerve avulsion (Ström, unpublished).

Glial cells are active players in the response to nerve injury. Roughly 2 days after injury, microglia become activated, start to proliferate and migrate to the site of the lesion, likely as a result of signals originating from the damaged neurons. This is thought to contribute to the denervation of lesioned neurons, which would indicate an important part in the synaptic stripping process. Microglia also display an inflammatory phenotype, with up-regulation of MHC class II, complement receptors, cytokines and complement components. At the end stage of activation, microglia become highly phagocytic and aid in clearing cellular debris from dying neurons. Several reviews have been written on the microglial response to nerve injury, see for instance (Aldskogius and Kozlova, 1998; Gehrmann et al., 1995).

Compared to the microglial response, the astrocytic reaction is somewhat delayed. Astrocytes do not proliferate, but become hypertrophic, migrate to the site of injury and start to express glial acidic fibrillary protein (GFAP) (Kozlova, 2003). Within weeks, astrocytes form a tight glial scar which insulates the injured area. An increase in neurotrophic factors, for instance platelet derived growth factor (PDGF) is also

observed, indicating regenerative and/or protective actions by astroglia (Aldskogius and Kozlova, 1998).

Although the response to injury follows a stereotypic pattern, genetic factors influence the degree of glial activation and neuronal loss. MHC class II and cytokine expression, as well as neurodegeneration, was shown to be regulated by non-MHC genes in a panel of inbred rat strains subjected to ventral root avulsion (VRA), one of the nerve injury models used in this thesis (Piehl et al., 1999). Strain dependent differences in astrocytic activation, MHC class I expression and T cell infiltration have been seen also in the second model studied here, i.e. facial nerve injury in the mouse (Ha et al., 2006; Lidman et al., 2002). There are few publications focused on mapping the genes which influence nerve injury response, but loci regulating neurodegeneration, T cell infiltration and MHC class II expression have been identified (Lidman et al., 2003).

3 ANIMAL POPULATIONS IN GENETIC RESEARCH

As previously mentioned, gene variants which regulate complex diseases are thought to have limited effect and may not be carried by all affected individuals, which makes the identification of these genes challenging. The two main strategies for mapping disease related genes are association studies and linkage analysis. Both approaches rely on the presence of DNA sequence polymorphisms, and linkage disequilibrium (LD) between a known genetic marker and a disease causing polymorphism in a gene. LD occurs when two particular alleles are inherited together more often than would be expected by chance. The sequence polymorphism most commonly used in genetic mapping today is the SNP, in which a single basepair is substituted for another, but microsatellites are also useful markers. Microsatellites are relatively short repetitive sequences, with variable repeat numbers between animal strains or individuals.

Association studies are performed in patient and control cohorts, and compare the frequency of one or more alleles in the two groups. Previously, association studies were performed mainly on already identified candidate genes, but rapid technical advances have enabled a more unbiased approach with whole genome association studies (GWAS), where SNPs across the genome are genotyped with chip technology.

Linkage analysis tracks inheritance of gene variants together with disease within families. This method has been successful mainly in the genetic mapping of Mendelian diseases, since disease causing genes in these disorders exert such a strong effect. Even so, decreased penetrance and/or multiple disease modifying genes have made linkage mapping also of monogenic disorders difficult. Due to the vast sample size needed to map complex disease genes, linkage studies of complex common disorders in human families have mainly yielded disappointing results (Altshuler et al., 2008).
In contrast, it has proven fruitful to use linkage analysis in animal populations in the efforts to identify genetic factors that influence disease (Becanovic et al., 2004). By crossing inbred rat strains that show different degree of susceptibility in relevant disease models, one can generate a backcross (BC), an F2 generation, advanced intercross lines (AIL) or congenic strains (illustrated in figure 3), in which it is possible

to investigate genetic regulation of disease phenotypes. The advantages of animal studies are several: decreased genetic heterogeneity, controlled environmental influence and large sample size.

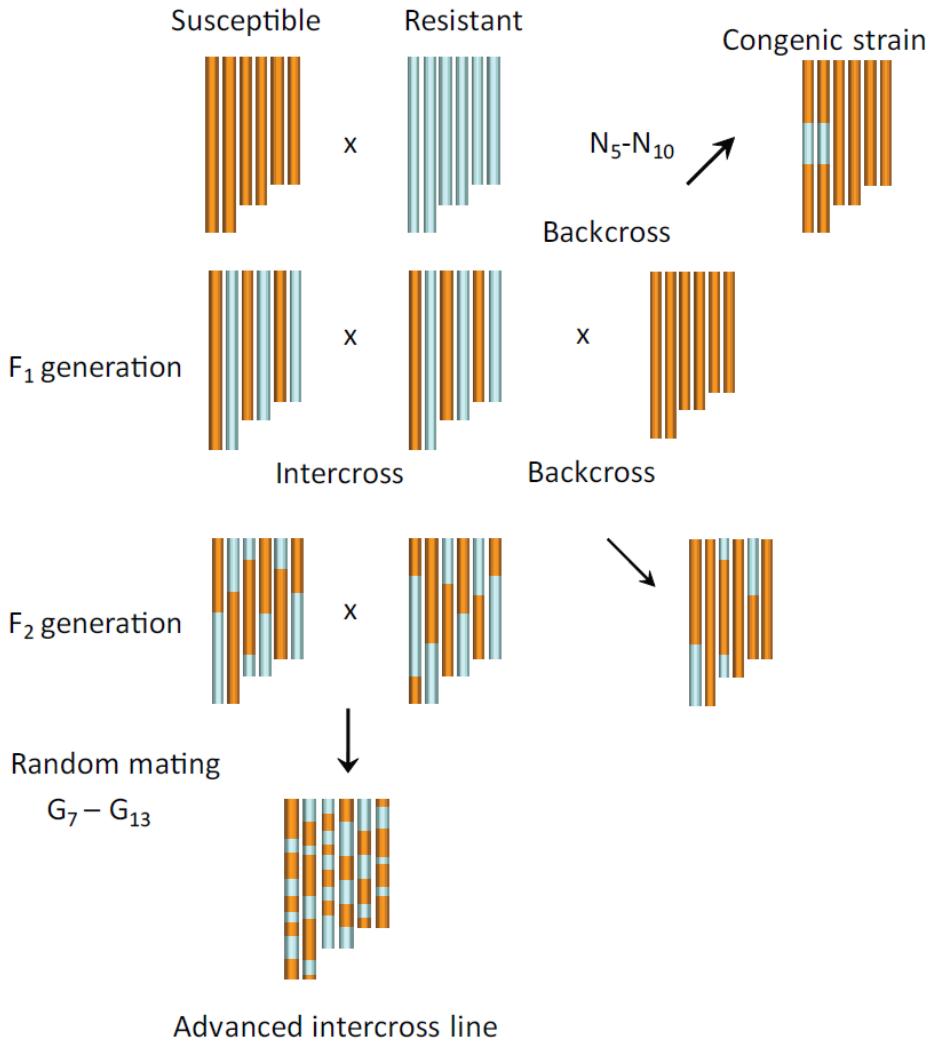

Figure 3. Schematic illustration of breeding strategies in experimental animal strains.

F2 and BC populations are suitable for whole genome linkage studies. The animals are used in the disease model of choice, and the phenotype of each individual is recorded. The phenotypes may include dichotomous traits such as disease or no disease, as well as continuous characters like disease severity or gene expression levels. The entire genome of each individual is then genotyped, using evenly spaced genetic markers at

a known position. By using computer software, such as R/qtl, it is possible to calculate the probability of linkage between certain genotypes at the marker position and the recorded phenotype. Regions that are linked to complex traits are called quantitative trait loci, or QTL. The process of QTL detection and fine-mapping is schematically depicted in figure 4.

Due to the limited number of generations, a drawback of the F2 or BC study is the few recombination events. This means that any QTL detected is likely to be very large, often comprising hundreds of genes. A good strategy to narrow down a region of interest is therefore to map it in an AIL. An AIL is generated by continuous breeding from the F2 population, for a further 6-10 generations. Inbreeding is limited by avoiding brother-sister mating. With each generation, the recombination rate is increased, which yields great possibilities of more precise mapping of a QTL. A gene region detected in a F2 generation can be reduced five-fold by intercrossing for an additional 8 generations. However, after the 10th generation, the gains are negligable (Darvasi and Soller, 1995).

Congenic strains are created by backcrossing an animal carrying the desired genotype in the locus of interest onto the parental for several generations. 10 backcrosses theoretically results in less than 0,1% donor contamination outside of the desired fragment. Congenic animals are valuable tools for confirming the effect of a QTL or gene. They can be tested in different disease models, to confirm or deny regulation of multiple clinical phenotypes, or be used for functional studies. Continuous backcrossing also enables development of recombinant strains, with very small inserts of the donor fragment, which allows for extreme fine mapping of genes.

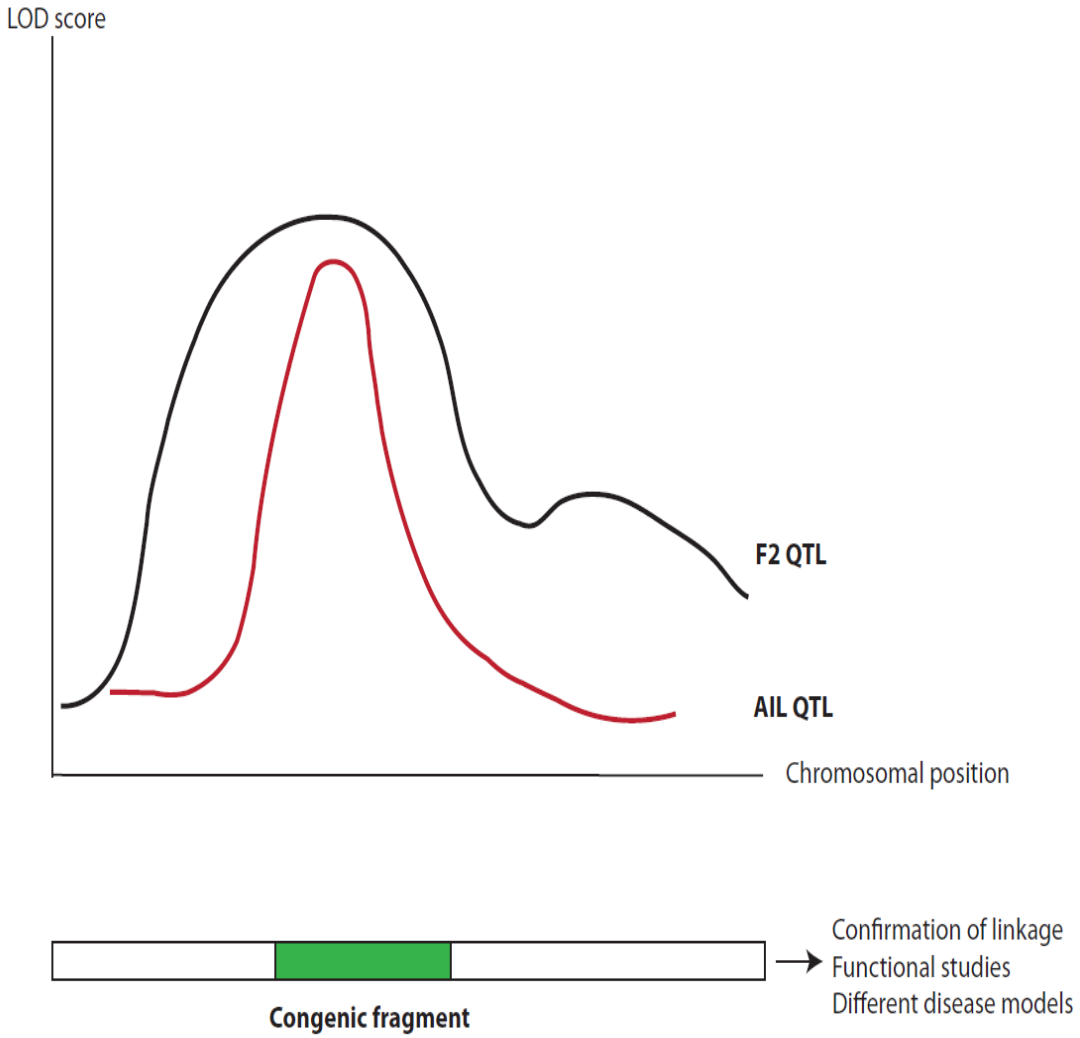

Figure 4. Simplified illustration of how a QTL is identified and fine-mapped in experimental crosses. AIL studies enable the QTL to be narrowed down, while congenic strains are suitable for functional studies.

4 AIMS OF THESIS

The overall aims of this thesis were to investigate genes and gene regions which affect inflammation and cell death in the central nervous system. More specifically, the aims were:

- To study MHC class II regulation by *Mhc2ta* and other genes in mouse and rat
- To study the role of *Mhc2ta* in autoimmune neuroinflammation
- To fine map genetic regulation of neurodegeneration after nerve injury in the rat

5 METHODOLOGICAL CONSIDERATIONS

The techniques used in this thesis are described in detail in each respective paper. In this section, different aspects regarding some of the methods employed will be discussed.

5.1 ANIMAL STUDIES

5.1.1 Animal populations

In this thesis, inbred rat and mouse strains have been used to study genetic regulation of neuroinflammatory and neurodegenerative phenotypes. In papers I and IV, the DA and PVG1^{1av1} parental rats, as well as congenic and advanced intercrosses between these strains, were used to map genetic regulation of the later VRA response and EAE. In paper II, a panel of inbred parental mouse strains, as well as a congenic strain created from C57BL/6J and 129X1/SvJ mice were used to investigate the response to facial nerve injury. In paper III, the early microglial response to VRA was characterized in an F2 cross between Brown Norway (BN) and LEW^{1N} rats, and the corresponding parentals.

For an overview of the different animal populations, please refer to figure 3 in the Introduction.

5.1.2 VRA

VRA is a standardized, highly reproducible model, where the roots of the L3-L5 segment are pulled from the spinal cord (fig 5A). This results in a proximal lesion, at the very border between the CNS and peripheral nervous system (PNS), which leads to neurodegeneration, local inflammation with glial activation, MHC class I and II expression and, to a certain extent, influx of T lymphocytes (Piehl et al., 1999). It should be noted that VRA is not aimed at mimicking a clinical disease phenotype, but rather provides an opportunity to characterize local neuroimmune and neurodegenerative processes.

5.1.3 Facial nerve injury

The facial nuclei contain motoneurons which control facial and whisker movements in rodents. Transection of the facial nerve (illustrated in Figure 5B) results in motoneuron loss in the facial nucleus, which coincides with T cell infiltration, and up-regulation of MHC antigens and cytokines (Olsson et al., 1992; Raivich et al., 1998). While VRA can be considered to be a purely central model, facial nerve injury is a more peripheral, and thus milder model with signs of regeneration of damaged neurons (Moran and Graeber, 2004).

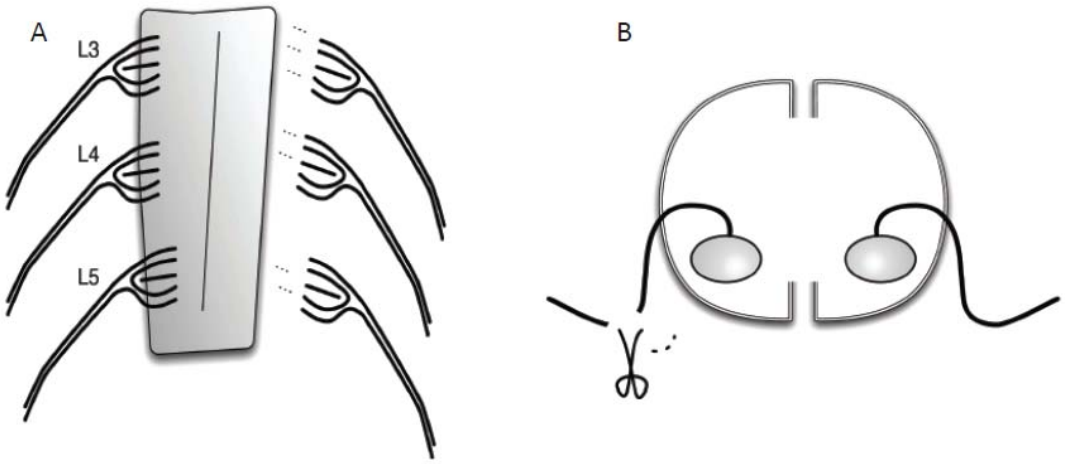

Figure 5. Schematic illustration of the nerve injury models used in this thesis. In VRA, the ventral roots are pulled from the L3-L5 segments of the spinal cord (A). In facial nerve injury, the facial nerve is transected (B).

5.1.4 Experimental autoimmune encephalomyelitis (EAE)

In this thesis, recombinant MOG in Incomplete Freund's adjuvant (IFA) was used to induce EAE, i.e. the animals were not immunized with mycobacteria. MOG is emulsified in PBS and IFA, and injected subcutaneously at the tail base of anesthetized rats.

Monitoring of clinical symptoms started at day 8 after immunization, and the rats were weighed and scored daily until the end of the experiment. The disease course is monitored daily by using a 5 step scale: Limp tail (1), wobbly gait (2), hind leg paralysis (3), front and hind leg paralysis (4) and moribund/dead (5).

5.1.5 IFN-γ stimulation, *in vivo* and *in vitro*

In vitro IFN-γ stimulation induces MHC class II expression in a variety of cells, including microglia (Panek and Benveniste, 1995). Direct injections of IFN-γ into the brain have likewise been proven to generate production of MHC class II antigens on microglia (Wong et al., 1984). In Paper I, we tested the effect of allelic differences in *Mhc2ta* both *in vitro,* by stimulating APCs with different doses of IFN-γ, and *in vivo* by intrastriatal injections of IFN-γ into brains of *Vra4*/*Mhc2ta* congenic rats. The outcome of these stimulations was analyzed with immunohistochemistry and flow cytometry, techniques which are discussed below.

5.2 EXPRESSION ANALYSIS

5.2.1 Real-Time PCR

In Real-time PCR, complementary DNA (cDNA) made by reverse transcription (RT) of RNA, is detected and quantified in "real time". The reaction mix contains a fluorescent dye, such as SYBR® Green which intercalates with double-stranded, but not single-stranded DNA. Upon binding DNA, SYBR® Green emits a strong fluorescent signal that intensifies as the PCR product accumulates. With each reaction cycle, the amount of DNA will double, eventually reaching an exponential amplification phase. Depending on the starting amount of material, the number of reaction cycles required to reach this phase will vary, thereby giving a measurement of expression. In order to quantify the amount of PCR product in each sample, the samples can be compared to a standard curve obtained by using a series of dilutions of the cDNA. In order to compensate for differences in, or small amounts of starting material, a housekeeping gene is used for normalization. This is a molecule considered to be expressed and degraded at constant levels in the tissue. However, the expression levels of housekeeping genes may be affected by inflammation, oxidative stress or other factors (Covacu, unpublished), which could give misleading results. Therefore, it is advisable to use two or more housekeeping genes, and to use housekeeping genes that are not affected by the model.

Real-Time PCR is a very sensitive method that detects very small changes in mRNA expression. However, it does not give information about which cell types produce the

mRNA. In addition, increased mRNA levels do not always lead to increased protein expression. Therefore, complementary expression analyses are needed.

5.2.2 Immunohistochemistry

Gene expression can be measured on the protein level by using immunohistochemical methods, where tissue sections are incubated with primary mono- or polyclonal antibodies specific for the protein of interest, visually detected by fluorescently labeled secondary antibodies binding the primary antibodies. Because mRNA levels aren't always correlated to the amount of protein synthesized by the cell, immunohistochemistry makes a good complement to the Real-Time PCR technique. Apart from quantifying the amount of protein, immunohistochemical staining can be used to identify the cellular location of a protein, or to study the regulation of protein in specific cells types.

5.2.3 Flow cytometry

Flow cytometry is based on the specific light scattering, excitation and emission by fluorescent molecules. The technique measures fluorescence per cell or particle, which allows for analysis and characterization of subpopulations of cells in culture, as well as the quantitative measurement – expression analysis – of the surface marker itself.

In paper I of this thesis, flow cytometry was used to analyze MHC class II expression on different subsets of rat APCs following IFN-γ stimulation. Monocytes and B cells were sorted from spleen cells, by capturing macrophages with antibodies to surface marker CD11b (note that the paper erroneously states that CD11c was used as a macrophage marker) and B cells with B220 antibody. Bone marrow derived plasmacytoid and myeloid DCs (pDC and mDC, respectively) were distinguished by their differential expression of CD11c and B220, respectively. OX-6 was used as a marker of MHC class II.

5.3 NERVE CELL COUNTS

In order to determine the degree of nerve cell loss, we use cresyl violet to stain tissue sections of the lesioned spinal cord. A minimum of 15 sections from each animal are stained and counted, and the mean value is calculated. The unlesioned side is used as

an endogenous control, and the number of surviving motoneurons on the ipsilateral side is compared to that of the contralateral side. The motoneurons are identified based on their morphology and location in the ventral horn. Cells lacking a visible nucleus and nucleolus in lateral motor nuclei are excluded.

The injury will induce morphological changes in the cells, including atrophy of the cell soma, so that the soma size on the lesioned side is roughly 72% of those on the uninjured side. These differences are accounted for by using the Abercrombie formula to adjust the counts: [N=n(T/T+D)], where N = true number of cells, n = expected number of cells, T = thickness of the section and D = diameter of the soma.

5.4 STATISTICS

5.4.1 Linkage analysis

As mentioned in the Introduction, linkage analysis is based on the inheritance of a genetic variant together with a certain phenotypic trait, i.e. the aim is to connect genotype to phenotype. There are several ways of doing this. The simplest method is the single marker analysis, where phenotype values between the groups of individuals stratified on their marker genotype are compared with t-test, analysis of variance (ANOVA) or any non-parametric test. Because the probability that a QTL is located at the exact position of a genetic marker is very low, it is more informative to employ interval mapping, where the genotype effect both at and between markers is included. This is done by analyzing several markers together, predicting genotypes between markers based on recombination frequency (Lander and Botstein, 1989). The results of a linkage study are presented as a logarithm of odds, LOD, which is the likelihood of QTL presence given the data available, vs. the likelihood of no QTL presence, given as log value with the base of 10. Thus, a LOD score of 3.0 means that a QTL linked to the measured phenotype is 1000 times more likely than not to be located at that position.

Depending on the nature of the dataset to be analyzed, different statistical models can be more or less appropriate. Thus, non-normally distributed data require a different model than normally distributed data, etc. It is therefore advisable to determine which is the most useful for each particular dataset. A drawback with

choosing a method based purely on the data is the limited possibility to perform additional analyses which are only available in some models, such as multiple QTL detection. Testing that similar results are obtained with a "less appropriate" model allows for incorporation of complex analyses.

In this thesis, the R/qtl software (Broman et al., 2003) was used for QTL detection in Papers III and IV. This software allows for linkage analysis with several models, both for single and multiple QTL mapping. For our purposes, the imputation model was most appropriate, since this method enables analysis of covariates, additive effects and epistasis (Sen and Churchill, 2001). Imputation also has the advantage of compensating for missing genotype data, by imputing the missing genotype based on the genotype of the nearest markers.

5.4.2 Significance threshold values

In order to determine if a LOD score is statistically significant, a threshold value needs to be calculated. There are various methods of doing this, depending on the population that is being analyzed. In the F2 generation, performing multiple permutation tests is an appropriate way of estimating genome wide significance levels. This model generates random genotype-phenotype sets and calculates LOD score accordingly. The process is repeated 1000-10 000 times (Churchill and Doerge, 1994) and the threshold represents the LOD score higher than that achieved by random chance in 95% of the permutations.

However, this approach is not recommended when analyzing an AIL population, which has a radically different and more complex family structure than does the F2. An alternative strategy is to calculate family residuals. The average phenotypic value of each litter is subtracted from the value of each individual animal of that litter. The analysis is repeated on the residual data, and the LOD score achieved is used as the threshold for the within-family effect. That way, any possible effect conferred by family is corrected for (Marta et al., 2009).

In this context it's relevant to point out that when fine-mapping an already existing QTL, the threshold of significance becomes of less importance than when identifying novel regions.

5.4.3 Confidence intervals

Finally, a confidence interval (CI) needs to be derived. This is an estimation of the location of the QTL. A 95% CI means that there is a 0.95 probability that the true location of the QTL is within the boundaries of the CI.

A commonly used method of estimating a CI is the LOD drop, or LOD support, method. The positions that correspond to a 1.5-2 LOD decrease from the maximum are set as the CI boundaries (Dupuis and Siegmund, 1999). In Paper IV of this thesis, a Bayesian credible interval was calculated, to provide additional support for the LOD support interval. Bayesian statistics finds the interval by estimating the probability of the hypothesis based on a prior probability that is updated through a series of re-evaluations of the existing data. This is an appropriate approach when analyzing an AIL cohort, since it does not require F2 family structure, in contrast to, for instance, the bootstrap method.

"Experience is the name everyone gives to their mistakes" – Oscar Wilde

6 RESULTS AND DISCUSSION

6.1 REGULATION OF MHC CLASS II AND AUTOIMMUNE NEUROINFLAMMATION (PAPERS I-III)

Previous studies in F2 crosses and AILs have revealed regulation of MHC class II expression after nerve injury by the *Vra4* region on rat chromosome 10 (Swanberg et al 2005, Lidman 2003). In paper I, congenic rats (DA.PVG^{1av1}-*Vra4* and PVG^{1av1}DA-*Vra4*, respectively) were used to confirm the effect of *Vra4* after VRA, and to investigate possible regulation of MHC class II expression on microglia and APCs after IFN-γ stimulation. The effect of *Vra4*/*Mhc2ta* on clinical disease was also investigated by using the congenics in EAE experiments. Paper II focuses on the effect of the *C2ta* gene after facial nerve injury in a panel of inbred mice, as well as in congenic mouse strains, while paper III investigates gene regions outside of *Mhc2ta* which regulate MHC class II expression in the rat.

Papers I and II both show an effect of *Mhc2ta* on MHC class II expression in the CNS after axonal injury. In paper I, results from the VRA experiments show a differential expression of both *Mhc2ta* and *Cd74*/MHC class II in the congenic animals compared to their respective parental strain, as revealed by both Real-Time PCR and immunohistochemistry. Interestingly, the PVG^{1av1}DA-*Vra4* strain had an almost two-fold up-regulation of *Mhc2ta* and *Cd74* compared to the DA strain, as analyzed with Real-Time PCR. This could be due to interactions with other genes in the PVG^{1av1} background or a lack of inhibition in the PVG genome that may be present in the DA rat.

In paper II, analysis of seven inbred mouse strains (C57BL/6J, DBA/2J, 129X1/SvJ, BALB/cJ, SJL/J, CBA/J, and NOD) reveal differential regulation of *C2ta* and MHC class II after facial nerve injury. Immunolabeling shows that MHC class II expression is largely restricted to microglia. Sequencing of the *C2ta* regulatory region in 4 of the strains, C57BL/6J, DBA/2J, SJL/J and 129X1/SvJ, reveals the presence of SNPs in promoter I and III where 129X1/SvJ differs from the other strains in the pI region and DBA/2J in the pIII region, respectively. Expression analysis of the individual isoforms showed that

the strain expression pattern for pI matched the pI haplotype. This was not seen for pIII where DBA/2J differed from the other strains with regards to genotype, but had a phenotype similar to the 129X1/SvJ and SJL/J strains. Finally, the effect of *C2ta* was tested in C57BL/6J/129X1/SvJ (B6/129) congenic strains. Expression analysis of this strain, along with C57BL/6J and 129X1/SvJ parentals, showed that the congenics had a phenotype similar to that of 129X1/SvJ with regards to *C2ta*, *Cd74* and pI expression. Taken together, these results strongly point to *C2ta* as the main regulator of microglial MHC class II expression after nerve injury. Furthermore, we see that the regulation in mice is restricted to the pI promoter of *C2ta*.

In addition to the VRA experiments, regulation of MHC class II by the *Vra4* region after IFN-γ stimulation was investigated both in the CNS and in subsets of APCs from DA and DA.PVG^{1av1}-*Vra4* rats (paper I). Intrastriatal injections of IFN-γ resulted in a much weaker MHC class II induction in the DA.PVG^{1av1}-*Vra4* strain compared to DA parentals, as measured with immunohistochemistry. Staining with OX-42, a marker for microglia, shows that the morphology and distribution of OX-42 and class II positive cells are similar, indicating that microglia are the main source of MHC class II. IFN-γ inducible MHC class II expression was also investigated in various antigen presenting cells; B cells and macrophages from spleen and myeloid and plasmacytoid dendritic cells from bone marrow of DA and DA.PVG^{1av1}-*Vra4* rats. FACS analysis of these cells showed that the most pronounced difference in class II expression between the strains is visible on B cells, while there is a trend towards decreased expression in macrophages, mDC and pDC from the congenic strain compared to those from the parental.

EAE experiments performed in paper I show a decreased incidence, cumulative EAE score and mean weight in DA.PVG^{1av1}-*Vra4* rats compared to DA parental, suggesting that quantitative differences of MHC class II are relevant for autoimmune disease. While it is likely that variations in the *Mhc2ta* gene are responsible for the differences in MHC class II expression, it is important to note the possibility that other genes within the *Vra4* region affect clinical EAE symptoms. For instance, CLEC16A (KIAA0350) has recently been identified as a candidate gene for several autoimmune disorders, including MS (Hafler et al., 2007), Crohn's disease (Marquez et al., 2009) and Type 1

Diabetes (Zoledziewska et al., 2009). This gene is situated in close proximity to the *Mhc2ta* gene in rat, human and mouse. It is therefore possible that this gene regulates the differential EAE phenotypes seen in Paper I, rather than *Mhc2ta*. Preliminary data, however, show no differences in expression of *Clec16a* in *Vra4* congenic rats after VRA (Harnesk, unpublished). Furthermore, there were no significant differences in expression of this gene in lymph nodes from MOG immunized DA and PVG rats (Thessen Hedreul et al., 2009). The precise role of *Clec16a* in disease therefore remains undefined.

In paper III, we set out to map gene regions outside of *Mhc2ta* that regulate MHC class II in a F2 cross between BN and LEW^{1N} rats. These strains differ in the early response to VRA with regards to MHC class II expression, in spite of the fact that they share the same MHC haplotype (RT1N) and several identical SNPs in the *Mhc2ta* region (Lundberg et al., 2001; Swanberg et al., 2005).

Linkage analysis in the F2 population revealed two main QTLs that regulate class II expression: *Neuinflam4* on chromosome 1 and *Neuinflam5* on chromosome 7, while there was no linkage to the *Vra4* region. In addition, there was evidence of epistatic interaction in the regulation of MHC class II.

Interestingly, higher MHC class II levels were promoted by the BN allele in *Neuinflam4*, and by the LEW^{1N} allele in *Neuinflam5*. This is interesting in light of the results from the congenic studies in paper I, where introgression of the *Mhc2ta* allele from the DA rat into the PVG^{1av1} genome led to an even higher MHC class II expression than in the original DA parental strain.

6.1.1 Is there a functional relevance of MHC class II levels?

MHC class II expression on APCs is crucial for T cell activation and clonal expansion, and the HLA locus is well established as a risk factor for autoimmune disorders. A general hypothesis is that genetic polymorphisms result in variations of the MHC molecule which may affect binding affinity and presentation ability of self antigens, and/or T cell interaction, in turn influencing disease (Gregersen, 1989; Nepom and Kwok, 1998). However, there has been little research on the functional effect of quantitative differences in MHC class II expression on autoimmune disorders.

Papers I and II show that *Vra4/C2ta* regulates MHC class II expression in microglia after nerve injury and in peripheral APCs after IFN-γ stimulation. Paper I also suggests a role for *Mhc2ta* in rat EAE, where *Vra4* alleles associated with lower MHC class II expression protected from disease. This indicates a possible effect of MHC quantity, as well as quality, on autoimmune neuroinflammation.

In order for a T cell to become activated, an APC needs to express only sparse amounts of MHC class II:peptide complexes (Irvine et al., 2002). It is tempting to speculate that even a small increase in MHC class II on APCs leads to a stronger T cell response with a higher degree of clonal expansion, which in turn could initiate, prolong or exacerbate disease. Treatment of EAE with statins, a group of drugs which among other things suppress *Mhc2ta*, leads to increased myelin repair (Paintlia et al., 2009) and decreased infiltration of immune cells to the CNS (Nath et al., 2004), results which may support this theory. Alternatively, expression levels of MHC class II may affect the cytokine profile of T cells, and in the long run have downstream effects on the immune response. Data from Baumgart and co-workers (1998) show that higher MHC class II levels on bone marrow-derived macrophages do result in differential cytokine expression in mouse T cells (Baumgart et al., 1998). This study included several different MHC haplotypes, however, and the results may therefore reflect an effect of MHC quality rather than quantity. It is also possible that the combination of a certain MHC class II molecule with increased expression through CIITA contributes to variations in T cell response, and in the context of disease, to an improperly regulated immune activation and autoimmunity.

Another line of thought is the relevance of MHC class II quantity in the thymus during development of central tolerance. In this context, a lower degree of MHC class II may lead to escape of auto-reactive T cells from the thymus to the periphery due to less effective presentation of self antigens, and thereby lead to autoimmune disease. Indeed, the disease associated SNP in the pIII promoter of MHC2TA described by Swanberg et al resulted in lower levels of MHC class II, although in peripheral blood (Swanberg et al., 2005).

In papers II and III, we show an increase in MHC class II in microglia after nerve injury. Does cell specific expression of immune related molecules, and immune activation in

the target tissue, matter in the context of disease? In RA, the role of the target tissue in disease progression has yielded increasing interest, since it has become known that resident fibroblast-like synoviocytes promote tissue destruction and inflammation in the joint, as reviewed by (Noss and Brenner, 2008). Emerging data indicate that a similar situation may be present also in CNS disease. For instance, mice unable to express the chemokine factor CCL2 in the CNS are protected from EAE (Dogan et al., 2008). Additional data which suggest that microglial expression of MHC class II is of importance for CNS disease development was recently provided by Suter and co-workers. By using bone marrow chimeras of mice lacking the pI isoform of *C2ta*, it was illustrated that MHC class II expression in microglia, macrophages and DC is dependent on this promoter. Furthermore, failure of these cells to express MHC class II lead to decreased risk of initiating EAE (Suter, 2008).

6.2 REGULATION OF OTHER INFLAMMATORY MARKERS (PAPERS I-III)

In both papers I and paper II, we measured expression levels of various inflammatory markers in the CNS after nerve injury in parental and congenic strains. While there was a difference in expression of microglial markers, T cell infiltration, MHC class I and co-stimulatory molecules between parental strains, we could see no effect of the *Vra4*/*C2ta* region on these phenotypes in the congenics.

The specificity of *C2ta* has been the target of some debate. Some studies do show an effect of *C2ta* on Th1/Th2 balance; however, many of these studies were performed in vitro and/or knock-out animals and may not reflect a situation with naturally occurring polymorphisms with a more modest impact (Itoh-Lindstrom et al., 1999; Patel et al., 2004; Zhou et al., 2007). Data presented by other groups indicate that *C2ta* does not have pleiotropic effects, but regulates a limited number of downstream targets, most of which are associated with antigen presentation (Krawczyk et al., 2008; Otten et al., 2006). The results presented here indicate that MHC class II is the principal target for *Vra4/C2ta*, and that *Vra4/C2ta* does not influence glial activation or expression of other inflammatory molecules in the CNS.

In paper III, genetic regulation of various other aspects of inflammation was investigated in addition to MHC class II. *Neuinflam4* and *Neuinflam5* also displayed suggestive linkage to complement factor 3 (C3), aside from MHC class II expression.

Two other main QTLs which each regulate several inflammatory molecules were detected; *Neuinflam9* on chromosome 10, linked to complement factor 1q (*C1q*), *Il1b* and *Cd11b*, and *Neuinflam10* on chromosome 11, linked to *C3*, *Cd11b* and *B2m*. The fact that several transcripts are regulated by the same region points to a common, upstream regulatory effect. Linkage strength varied for the different inflammatory markers. This effect may be specific for this particular cross, but could also reflect a smaller genetic impact on the expression of these molecules. Interestingly, the BN and LEW^{1N} parental strains do not differ significantly in the expression of several of the studied molecules, yet a genetic regulation of these markers could be detected in the F2 offspring.

6.3 REGULATION OF NEURODEGENERATION BY *VRA1* AND *VRA2* (PAPER IV)

The aim of paper IV was to finemap and verify previous findings regarding *Vra1* and *Vra2,* which were linked to neurodegeneration in a F2(DAxPVG^{C}) intercross, by using the G8 and G10 of a DAxPVG^{1av1} AIL, and a panel of congenic strains.

In both AIL cohorts, *Vra1*, located on chromosome 8, displayed linkage to neuronal loss, 14 days after VRA, thus reproducing the results from the F2 study. However, when the data from the AIL populations were combined, linkage strength was substantially increased. This also reduced the *Vra1* region to roughly 13 Mb.

The effect of *Vra1* was also investigated in three congenic rat strains, 21 days after VRA: DA.PVG^{1av1} -*Vra1*-R1, DA.PVG^{1av1} -*Vra1*-R2 and DA.PVG^{1av1} -*Vra1*-R3. DA.PVG^{1av1} -*Vra1*-R1 and DA.PVG^{1av1}-*Vra1*-R3 showed a significantly decreased degree of neurodegeneration compared to the DA parental strain, whereas DA.PVG^{1av1}-*Vra1*-R2 retained the DA phenotype. These results indicate further that the gene(s) regulating neuronal loss is located within DA.PVG^{1av1}-*Vra1*-R1 and –R3. *Vra1* is thereby reduced further, to comprise an interval of 9 Mb.

In the original F2 study, a suggestive QTL on chromosome 5, denoted *Vra2*, was linked to neurodegeneration. In paper IV, *Vra2* again showed a weak linkage to nerve cell loss in G10, but not in G8. However, a two-dimensional scan showed an additive effect between *Vra1* and *Vra2*, with a highly significant effect from *Vra2,* suggesting that although the effect of *Vra2* alone is weak, this region is of importance for the degree

of nerve cell loss. Because neurodegeneration reaches a peak at 21 days after VRA, the chance of capturing linkage of this region to nerve cell death would also be increased by sampling data at a later time-point than in the AIL experiments performed in paper IV.

To elucidate further the effect of Vra2, and the combined effects of *Vra1* and *Vra2*, another good strategy is to isolate this genetic fragment in *Vra2*- and bi-congenic strains, now being bred in our laboratory.

Nerve cell loss is a common feature of several CNS diseases, not only in primary neurodegenerative disorders such as AD and ALS, but also after ischemic stroke and during MS. The molecular pathways of neurodegeneration are not fully elucidated, but oxidative stress (Miller et al., 2009), dysregulation of stress (heat shock) proteins (Abdul et al., 2006), and excitotoxicity (Heng et al., 2009) have been proposed as mechanisms, see also (Bossy-Wetzel et al., 2004). The *Vra1* region now contains 59 genes, of which 14 are pseudogenes. This is too large a number to identify specific causative gene(s), or disclose pathogenic pathways. However, the gene list contains some interesting candidates, such as glutathione transferases A2 and A4 (Gsta2, Gsta4), heat shock protein Hsp90 and mitochondrial protein L41, all of which may be of importance for cell cycle regulation and/or cell survival.

VRA experiments in recombinant congenic strains, carrying very small inserts of the *Vra1* region, along with sequencing and expression analysis of genes located within these inserts are ongoing in our research group.

7 CONCLUSIONS

The results of this thesis show that inflammation and nerve cell loss in the CNS is subject to a complex genetic regulation, and that the genetic factors involved can be mapped and studied in animal models by using different breeding strategies and linkage analyses.

Specific conclusions that can be drawn from the results presented are:

- *Vra4/C2ta* regulates expression of MHC class II after nerve injury in mouse and rat, and after *in vivo* and *in vitro* IFN-γ stimulation in rat
- *Vra4* regulates susceptibility and severity of rat EAE
- *Vra4/C2ta* does not regulate expression of MHC class I, co-stimulatory molecules, T cell infiltration or microglial markers after nerve injury
- Outside of *Mhc2ta*, MHC class II expression is regulated by regions on rat chromosome 1 and 7
- *Vra1* regulates neurodegeneration after VRA, with additive effects by *Vra2*

8 FUTURE PERSPECTIVES

The results presented here provide new information about neuroinflammatory and neurodegenerative processes, but also generate new questions that remain to be answered.

The exact role played by *C2ta* and differential MHC class II expression in immune activation is still not defined. Characterization of this key immunological molecule is of outmost importance to increase our knowledge about inflammatory pathways. *In vitro* studies, where the effect of different degrees of MHC class II expression on e.g. T cell proliferation, subpopulations and cytokine profiles is investigated, may provide additional information on the downstream effects of high MHC class II levels.
In this thesis, the focus lies on inflammation and the role of *C2ta* in the CNS. It is of interest to test effects of *C2ta* in disease models other than nerve injury and EAE. The congenic mouse strains used in the present study will be included in future projects, with the aim of characterizing the role of *C2ta* in arthritis and atherosclerosis.

The regulation of neurodegeneration by *Vra1* needs to be elucidated. Preliminary data show that *Gsta4*, one of the genes within the *Vra1* locus, is differentially regulated in both spinal cord and primary microglial cultures from DA and PVG^{av1} rats (Ström, unpublished). The GSTA4 protein detoxifies 4-hydroxy-2-nonenal (4HNE) (Vaillancourt et al., 2008), a peroxidized lipid which has been implicated in neurodegenerative disorders, including AD (Reed et al., 2009) and ALS (Simpson et al., 2004). This makes *Gsta4* an interesting candidate in the regulation of nerve cell death.

The response to VRA is complex and regulated by multiple genes, as demonstrated by Paper III of this thesis. Global gene expression profiling of a F2(DAxPVG^{av1}) intercross has been performed and combined with genome wide linkage analysis. Further analysis of this data will help reveal important genes and pathways, as well as identify genetic networks involved in the neuroinflammatory and neurodegenerative process.

The animal strains described in this thesis carry commonly occurring genetic variants, rather than mutations which lead to defective or lacking gene products. This brings with it the advantage of more adequately mimicking the scenario of complex diseases, compared to for instance knock-out mice which may be described as analogous to Mendelian disorders or traits. Hopefully, the strains used here will be helpful in the efforts to shed more light on the complex processes of neuroinflammation and neurodegeneration, and perhaps also in studies of clinical disease.

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