GENETIC VARIABILITY COMMONLY AFFECTING NEURODEGENERATION AND NEUROINFLAMMATION

Mikael Ström
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To my family
ABSTRACT

Loss of nerve cells and axons is a common feature of common complex neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases. However, also Multiple Sclerosis (MS), primarily an autoimmune disorder, has a prominent neurodegenerative component. In complex disorders, many components affecting disease development and disease progression in combination make up the overall risk. In general, we divide these factors into inherited genetic factors and environmental factors. In addition, there are sometimes complex gene-environment interactions that make it difficult to identify individual risk components.

In this thesis, I have focused on a translational approach to find genetic determinants of nerve cell survival in a simplified experimental model of nerve injury-induced neurodegeneration. The aim has been to find novel genes/pathways whose relevance subsequently can be tested in human disease. Through various genetic mapping approaches I demonstrate a strong inverse correlation between neuronal survival and expression and protein levels of an enzyme involved in detoxification of certain oxidation by-products. This enzyme, Glutathione S-Transferase alpha 4 (Gsta4), is highly efficient in catalysing the reduction of the neurotoxic aldehyde 4-hydroxynonenal (HNE), which is generated during lipid peroxidation and has previously been implicated in the pathogenesis of various neurodegenerative disorders.

The relevance of this mechanism was also tested in a model of traumatic brain injury, where Gsta4 levels inversely correlate with degree of neuronal loss as well. In addition, rats with higher Gsta4 levels have a more favourable outcome after injection of HNE directly into the cortex. Taken together, these two studies provide strong support for the notion that the identified pathway is highly important for ability to cope with oxidative stress and in turn of relevance for nerve cell survival in different types of acute injury.

Finally, a possible role for Gsta4 is tested in experimental autoimmune encephalomyelitis (EAE), a model of MS. No discernible clinical effect was observed between congenic rats with higher Gsta4 expression and the parental strain. However, lower Gsta4 expression was associated with a stronger autoantibody response. Protein modifications by HNE have in other inflammatory models been documented to induce a stronger antibody response, which is consistent with the obtained results. Intrathecal antibody production is an important diagnostic marker in MS, and hypothetically the HNE pathway can play a role for disease course through both neurotoxicity and amplification of the immune response. This was tested in a large case control cohort of MS, where suggested associations both to clinical and immune phenotypes were found. In summary, the results presented encourage further studies on the Gsta4-HNE pathway both in conditions of acute nerve injury and autoimmune neuroinflammation.
De flesta har nog upplevt vad som händer med olja som blivit stående lite för länge hemma i köket; den härsknar. Den process som huvudsakligen ligger bakom detta är lipidperoxidering, d.v.s att fetterna reagerar med syre. Om man sticker ned en kvist rosmarin blir det inte bara gott, utan det förhindrar också att oljan härsknar. Rosmarin innehåller ovanligt stora mängder av särskilda molekyler som binder upp reaktiv syre och förhindrar reaktion med oljan; antioxidanter.

Kroppens celler använder syre för att tillgodogöra sig energi och innehåller dessutom stora mängder lipider, vilket gör dem känsliga för lipidperoxidering. Cellerna har därför utvecklat olika sätt att skydda sig, bland annat genom att likt rosmarnoljan innehålla antioxidanter som kan neutralisera oxidanterna och därmed förhindra att dessa reagerar med andra molekyler. Dessutom kan denna process ske än mer effektivt genom att det finns särskilda enzymer som katalyserar reaktionerna och förbättrar skyddet. Det centrala nervsystemet är särskilt känsligt för lipidperoxidering genom sitt höga syrebehov i kombination med ett högt fettinnehåll. Hjärnan, som endast upptar cirka 2% av kroppsvikten, konsumerar ändå 20% av allt syre i kroppen under vila. Nervcellerna har dessutom ett extra stort behov av att skyddas från skadliga effekter av lipidperoxidering eftersom de i princip inte alls kan nybildas.

Bakgrunden till detta avhandlingens arbete var observationen att två olika stammar av inavlade laboratorieråttor uppvisar en stor variation i antal överlevande nervceller efter en standardiserad nervskada under kontrollerade miljöbetingelser, vilket indikerar en ärfilig reglering av nervcellernas känslighet. Genom att i en första studie definiera ett genområde som styr denna skillnad och sedan i ett uppföljande arbete med olika experimentella metoder identifiera en enskild gen i området kan vi här visa på ett samband mellan enzymet Gsta4, som katalyserar oskadliggörandet av reaktiva biprodukter av lipidperoxidering, och nervcellöverlevnad. Dessa reaktiva biprodukter har påvisats i flertalet kroniska neurodegenerativa sjukdomar, men även vid akuta tillstånd som traumatisk hjärnskada. Det tredje arbetet visar att samma ärfliga variation i Gsta4 påverkar nervcellsdöd även efter experimentell hjärnskada.

Slutligen, i avhandlingens fjärde arbete, visas att ärfligt betingad variation i Gsta4 också påverkar det immunolgiska svaret i en modell för sjukdomen MS och att en liknande variation även kan föreligga hos människan. Sammantaget understryker de resultat som här presenteras betydelsen av oxidativ stress och lipidperoxidering vid olika sjukdomstillstånd i nervsystemet och identifierar ett enskilt protein, Gsta4, vars funktion är ärfligt reglerad och som påverkar nervcellsdöd. Vidare studier behövs för att i detalj kartlägga betydelsen av ärflig variabilitet i GSTA4 för olika former av neurodegenerativa sjukdomar hos människor.
LIST OF PUBLICATIONS


III. Faiez Al Nimer*, Mikael Ström*, Rickard Lindblom, Bo-Michael Bellander, Jens Randel Nyengaard, Olle Lidman, Fredrik Piehl Naturally occurring variation in the glutathione-S-transferase 4 gene determines neurodegeneration after traumatic brain injury Submitted manuscript

IV. Mikael Ström, Faiez Al Nimer, Rasmus Eurén, Melanie Thessen Hedrul, Ingrid Kockum, Mohsen Khademi, Tomas Olsson and Fredrik Piehl. Variability in Gsta4 is associated with intrathecal antibody responses in experimental autoimmune encephalomyelitis and suggested clinical and immune phenotypes in multiple sclerosis. Manuscript

*These authors contributed equally to the work
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LIST OF ABBREVIATIONS

AAO  Age At Onset
Aβ   Amyloid beta
AD   Alzheimer’s Disease
AIL  Advanced Intercross Line
ALS  Amyotrophic Lateral Sclerosis
ANA  Anti-Nuclear Antibodies
BC   Backcross
CI   Confidence Interval
CNS  Central Nervous System
CSF  Cerebrospinal Fluid
DA   Dark Agouti
EAE  Experimental Autoimmune Encephalomyelitis
EDSS Extended Disability Severity Scale
Elisa Enzyme Linked Immunosorbent Assay
GPx  Glutathione Peroxidase
GSH  Glutathione (reduced)
GST  Glutathione S-Transferase
Gsta4 Glutathione S-Transferase alpha 4
GWAS Genome Wide Association Study
HD   Huntington’s Disease
HNE  4-Hydroxynonenal
Ip   Intraperitoneal
LD   Linkage Disequilibrium
LOD  Logarithm Of Odds
LPO  Lipid Peroxidation
LRS  Likelihood Ratio Score
Mb   Megabase
MDA  Malondialdehyde
MOG  Myelin Oligodendrocyte Glycoprotein
MS   Multiple Sclerosis
MSSS Multiple Sclerosis Severity Score
OND  Other Neurological Disorder
PCR  Polymerase Chain Reaction
PD   Parkinson’s Disease
PUFA Polyunsaturated Fatty Acid
PVG  Piebald Virol Glaxo
QTL  Quantitative Trait Loci
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
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<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
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<td>VRA</td>
<td>Ventral Root Avulsion</td>
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1 AIMS OF THIS THESIS

The aims of these thesis was to investigate a previously identified gene region in the rat that was shown to affect the degree of neuronal survival after nerve injury, in order to find the underlying gene(s) and pin point a relevant mechanism. Further, to use a translational approach to explore whether this mechanism is relevant in other models and in human disease.

In detail, the aim for the different studies was:

Paper I: To reproduce and fine map the previous linkage to nerve cell death after nerve injury in advanced intercross lines. Further, to prove the effect of the linked region by the use of congenic strains.

Paper II: To position a likely candidate gene among the remaining genes in the fine mapped genomic region.

Paper III: To explore if the identified genetic effect is of relevance also in other models of neurodegeneration and, if so, dissect underlying mechanisms more in detail.

Paper IV: To explore if the identified genetic variation affects outcome in experimental autoimmune encephalomyelitis and its model disease, multiple sclerosis.
2 GENETICS OF COMPLEX DISEASE

2.1 OVERVIEW

The first diseases in which causing gene variants were discovered were so called monogenic, or mendelian, disorders where a single gene variant causes disease. Examples of diseases linked to monogenic traits include haemophilia, cystic fibrosis, sickle cell anaemia and Huntington’s disease. However, monogenic diseases are overall rare, although the number of different monogenic traits is large. Today approximately 3000 gene variants have so far been detected and these are suggested to make up 30-50% of all suspected mendelian disorders [1]. However, more common is a polygenic nature of disease, where a number of gene variants are involved in the pathogenesis and where each gene gives a small contribution to total disease risk.

A majority of the polygenic disorders are further likely to be associated with known or yet unknown environmental factors, that adds to risk or interact with the genetic factors, so called gene-environment interactions. These diseases are referred to as complex diseases, where gene variants that increase the risk are called predisposing genes. Predisposing gene variants generally only slightly increase the risk to develop a certain disease and therefore the variants also frequently occur in the healthy population. A certain gene variant can also be predisposing for one disease, but protective in other settings. There are for example studies that indicate that positive selection for genes involved in immune response to pathogens, protective for infectious diseases, that in the past was extremely favourable and positively selected, today turn out as risk genes for autoimmune disease [2]. Further, alleles that are defined as risk alleles in one autoimmune disease may be protective in another [3]. These factors result in that not only the disorders are complex, but also the genetic dissection of them.

2.2 THE THRESHOLD MODEL

The threshold model in a complex disease refers to the multifactorial predisposing factors that together add risk. Taken together, all these factors might result in that the total disease risk reaches above a level where disease is elicited. A low influence from predisposing genes might still result in disease development is there is a strong influence of environmental factors and vice versa. This is schematically depicted in figure 1. However, this picture is two dimensional and shows a purely additive scenario. More likely, 1+1≠2 and a combination of a certain genetic and environmental, or several genetic factors, might give a much higher total contribution than each contributing factor alone.
The main non genetic risk factor for neurodegenerative disorders, that seems to be common to almost all diseases, is increased age. Extremely generalized, early onset of neurodegenerative disorders seems to have a stronger genetic component, while late onset cases have less genetic influence and likely more influence from other factors. Early onset cases are more likely to occur in families, so called familiar cases, while late onset cases are more likely to be sporadic. Since neurons of the CNS do not renew, at least not to a large extent, and some loss over time is unavoidable there is a slow progression towards disease. However, under favourable conditions the slope is so low that disease will not be developed. However, early or continuous environmental exposure and/or genetic predisposition can lower the distance to disease, change the slope towards disease progression etc. (figure 1).

**Figure1**: The threshold model for developing complex disease, where the total disease burden of several genetic and environmental factors add on top of each other with individual pattern, and lead to disease development if the threshold level for total risk is exceeded (left). Environmental contribution will be added over time, and so also effect of genetic variation that might only have effect in the presence of certain environmental factors. Increased age is always a risk factor for neurodegenerative disorders, but without other factors disease will not develop [1] (right). Genetic factors, like lower capacity to reduce varying toxic molecules, might give a steeper slope under the same environmental exposure and lead to earlier disease development [2]. Certain factors, genetic or environmental, early in life can result in a lower spare capacity and disease development earlier in life [3].

2.3 GENETIC DISSECTION

A main challenge in genetic dissection of complex disease is that predisposing gene variant that only slightly contributes to increased disease risk frequently occurs also in the healthy population. The gene variant is said to have low penetrance. In addition,
contributions from a large number of rare genetic variants in the population might significantly affect risk at individual level, but have low relevance on population level. A given question based on these facts is if there actually is of any value to study genetics of complex diseases?

However, there are many reasons why genetic dissection of complex disorders is important. First of all, increased knowledge about risk factors can point out certain common molecular pathways of importance for disease, although different genes and variations might underlie the pathway changes. Drug development can focus on targeting the pathway rather than individual genes. Second, certain gene variants might reflect sub-phenotypes of the disease and also predict disease course and severity. This could be of importance at individual level for developing individually adjusted treatment. With a predicted aggressive disease course, a more efficacious treatment with more side effects might be acceptable than with a predicted milder disease course.

Knowledge about risk factors at individual level could also be used in preventive treatment or in order to actively avoid known environmental factors to reduce the risk of reaching above the threshold level, or extend the time before development of symptoms. There are already companies where a person can screen his/her genome for known predisposing genetic variants of common disorders and get advice from medical expertise on how to reduce the risk of developing these. This type of strategy can likely be successful in reducing disease prevalence in the future with better knowledge of disease aetiology, but at the same time raise many ethical questions of social and society aspects, which are necessary to discuss but will not be specifically dealt with here.

2.4 A TRANSLATIONAL APPROACH

This study primary looks at neuronal survival after induced mechanical injury in the rat. The final goal of this type of research is of course not to cure injured rats, but to find mechanisms of general relevance for neurodegeneration that can be translated into human disease. This type of translational research in animal models have been widely used and have improved our knowledge about several important disease mechanisms such as Cd36 in insulin resistance [4], Ncf1 and ZAP-70 in arthritis [5, 6], CTLA-4 in diabetes [7] and C5 in liver fibrogenesis [8] but also generated basic understanding of biological functions as iron uptake in red blood cells [9].
The study design in this thesis is based on a forward genetics approach, where an observed trait is studied in genetic context to identify the underlying variation and thereby generate an hypothesis for disease mechanism. This is in contrast to hypothesis driven research where the starting point is a suggested gene and mechanism which is manipulated and tested for phenotypic effect.

Hypothesis driven genetic research for candidate genes for human disorders have the disadvantage that the hypothesis has to be generated based on previous observations and therefore is less likely to identify novel mechanisms. A widely used unbiased approach is screens in large materials that cover the whole genome (GWAS, later described). However, a disadvantage here is the number of tests utilized, raising the threshold level for significance. The translational approach in genetic dissection combine the unbiased genetic screening with the hypothesis driven approach by testing identified hypothesis/candidate genes from unbiased animal studies in human disease.
3 NEURODEGENERATIVE DISORDERS

Genetics plays an important role in neurodegenerative disorders. There are examples of genetic variations that directly lead to disease (monogenic), but also likely a large effect on disease development and severity in complex disorders. The following section aims to give a brief overview about different diseases with a neurodegenerative component and current knowledge of the role of genetic background for disease development.

3.1 PRIMARY DEGENERATIVE DISORDERS

*Huntington's disease* (HD) is characterized by uncontrolled excessive motor movements, named chorea, and cognitive and emotional deficits. It was first described and named after George Huntington who published a paper that described the disease in 1872 [10]. Onset is typically seen in middle age, but can start at any age from infancy to old age and the disease is neuropathologically characterized by neurodegeneration in selective parts of the brain. HD has a dominant mendelian inheritance and was linked to an unstable triplet repeat expansion of a gene later named Huntingtin in 1993 [11]. The CAG expansion gives rise to a chain of extra glutamine amino acids in the protein, called PolyQ. The number of extra repeats largely affects the disease onset and account for approximately 60% of variance. 35 repeats or less is not associated with disease, while 36-40 shows incomplete penetrance [12] A common pathological pattern with many of the neurodegenerative disorders is formation of protein aggregates, here with mutant HTT and polyQ. However, studies implicate that the aggregates are not neurotoxic but rather that the mutant protein is in a dose dependent way [13].

*Alzheimer's disease* (AD) was first described in 1906 by the German pathologist Alois Alzheimer. He classified it as a rare disease with dementia mainly in people under 65. In 1976 Robert Katzman reclassified the disease with the findings that senile dementia in people over 65 in many cases share the same characteristics as previously observed in Alzheimer’s [14]. Still today, the age of 65 is used as discriminating the two forms early onset and late onset AD (EOAD and LOAD). Alzheimer’s disease is today the most common cause of dementia and approximately 26.6 million people had the disease in 2006 and it is further estimated to increase 4-fold by 2050 meaning that 1 person of 85 would live with the disease [15]. The disease is characterized by progressive loss of neurons in several brain regions. Histopathology shows intracellular formation of neurofibrillary tangles (NFT) made up of abnormally phosphorylated Tau protein together with formation of extracellular senile placks consisting of aggregations of misfolded amyloid-β (Aβ). The cause of the disease is still unknown but there is a strong genetic component in AD. Among early onset AD, linkage analysis in families has revealed linkage to rare mutations in the genes encoding APP [16], presenilin-1
(PSEN1) [17], and presenilin-2 (PSEN2) [18]. However, also in the later onset sporadic cases there is a strong genetic component and it is estimated that genetic components account for approximately 60-80% of the attributable risk [19]. There has been 19 GWAS studies conducted on AD and between 2007 and 2011 according to www.alzgene.org [20]. The strongest association, detected in all studies is to the apolipoprotein E (APOE) ε4 allele, which has been attributed to up to 30% of the risk of LOAD [21]. However, of all other genes detected in different studies, only four other can be considered as confirmed by the number of replications, which are Bridging integrator 1 (BIN1), Clusterin (CLU), Complement Component receptor 1 (Crl) and Phosphatidylinositol-binding clathrin assembly protein (PICALM) which all are related to production, aggregation or clearance of Aβ [22].

Parkinson's disease (PD) was described in 1817 by James Parkinson and the syndrome was later named after him. Although 200 years have passed, the cause of the disease is still unknown. The major and strong risk factor is age, with a steady increase in risk after the age of 60 [23]. There is no difference in risk between ethnic groups and the vast majority of cases are sporadic. In PD a specific group of neurons, dopaminergic nigrostriatal neurons, are progressively lost leading mainly to moving deficits. Neuronal loss is seen together with formation of intraneuronal Lewy bodies, which are aggregates of an abnormal form of the α-synuclein protein. There is still no established causative environmental factor although exposure to several pesticides that induce oxidative stress has been associated with increased risk [24] while smoking, in a variety of diseases regarded as a risk factor, is potentially protective as well as is caffeine (reviewed by [25]). Twin studies have indicated that early onset PD have a stronger genetic influence than late onset, which likely is more influenced by environmental factors [26]. Early onset PD can be linked to rare monogenic mutations, but accounts for only 5-10 % of the cases [27].

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that preferentially affects motoneurons in the spinal cord, brain stem and motor cortex. It was originally characterized and named by Jean-Martin Charcot 1874, although studies of symptoms and pathological changes had been presented by many others since at least 1830 [28]. It is a complex and heterogeneous disease with unknown cause and the pathogenesis is not fully understood. However, it includes inflammation and release of pro-inflammatory cytokines from microglia and astrocytes, as well as oxidative stress. ALS is often divided into familial ALS (FALS) and sporadic ALS (SALS). Approximately 5-10% of cases are familial and today there are 18 genes associated to this form where SOD1, important for oxidant defence, is the most well-known and accounts for approximately 20% of the FALS cases [29]. It also accounts for a few per cent of the sporadic cases, and in total 164 different mutations has been found in the gene of ALS patients [30]. Possibly, the genetic predisposition of FALS is stronger than expected due to low disease penetrance [31]. There are two main hypothesis in how mutant
SOD1 affects neurodegeneration: either through altered redox reactions or by toxicity of the mutant protein itself, since aggregates of mutant SOD1 is found in inclusions both in ALS and in disease models [32].

ALS more commonly affects men than women, but has an almost uniform distribution over the world, with one exception; Guam where it is 100-times more common. Genetic studies have not been able to find an explanation to this increase, thus focusing interest into environmental factors. For a long time, the neurotoxic amino-acid BMAA, which is produced by Cyanobacteria and found in cycad seeds commonly found and used at Guam, has been suggested to play a role for development of ALS and also other neurodegenerative disorders [33]. In contrast to most other disorders, slimness with low BMI and high degree of exercise (athletes) seem to be risk factors to develop disease [34]. An Italian study has pinpointed professional soccer players at being of particularly high risk [35, 36]. However, data has to my knowledge not been reproduced in other professional sport leagues.

### 3.2 TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is the leading cause of death and neurologic disability in young individuals in developed countries. Genetics of TBI differ from the above mentioned disorders in that there is no direct effect of genetics on disease onset, although it could be argued that genetic factors influencing behaviour could be a risk factor. The clinical spectrum of TBI is wide and obviously the most important factor affecting outcome is the type of trauma, which range from mild concussion to large hematomas: almost no injury is exactly similar to another. However, not only the type of injury affects outcome, but also gender and age are important factors [37-39]. Actually, a main established non genetic risk factor for poor outcome in TBI is advanced age.

Nerve cell death after TBI can be divided into primary damage directly caused by the trauma and secondary delayed damage that lead to additional neuronal degeneration. Secondary neurodegeneration is a process that can be on going for weeks to years after the injury. Rapid stabilization of vital functions is important to reduce secondary degeneration and improve repair. Hypoxia after TBI has been shown to increase the degree of neurodegeneration [40]. The molecular mechanism of secondary degeneration is not fully understood but pathophysiological processes like excitotoxicity, inflammation, apoptosis, oxidative stress and oedema formation seem to play a major role in the delayed neuronal death. From studies in other diseases where the same pathways are activated we know these pathways can be genetically regulated. Hence, it seems reasonable to hypothesize that inter-individual variation in secondary degeneration is likely to at least to some extent be genetically regulated. Although
extensive research has been conducted with many clinical trials in the field, there is still no efficient treatment to reduce secondary degeneration.

Little is known about genetics of SND severity after TBI, but APOE ε4, previously described as a risk gene for AD, has also been associated with worse outcome of TBI [41, 42]. In a large number of studies TBI has also been identified as a risk factor for later development of AD [43]. This is also the case in stroke, where SND plays an important role for cognitive decline and also this process resembles many molecular characteristics of AD [44]. The notion that a TBI earlier in life increase risk of AD reflects the theory of the threshold model, where TBI likely is a strong environmental factor adding risk and reduce the time span for developing (figure 1).

3.3 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a complex autoimmune disorder, which is characterized by chronic inflammation where the immune system attacks the myelin and oligodendrocytes that make up the myelin sheets around the axons in the CNS. In parallel, or as a consequence of demyelination, axons are damaged leading to disruption of neuronal signalling. The symptoms of MS varies widely depending on which signals are disrupted but typically includes muscle weakness, sensory and balance disturbance, visual problems and cognitive impairment.

An important diagnostic marker in MS is the presence of intrathecal antibody production. This is defined as presence of antibodies in the CSF that are uniquely synthetized by CNS resident B-cells. There are two measurements of intrathecal antibody production, presence of so called oligoclonal bands (OCB) and IgG-index. Presence of OCBs is defined as immunoglobulin bands identified by isoelectric focusing that uniquely exist in the CSF and not in plasma. IgG-index is a quantitative measure of IgG-levels where the index represents a quote between IgG in CSF versus serum that is normalized against the same albumin quote, thereby adjusting for barrier damage [45]. OCBs are present in approximately 95% of MS patients and a majority, 70%, also display elevated IgG index [46, 47].

MS was first described as an independent neurological disease by Jean-Martin Charcot in 1868, called sclerose en plaques[48]. Since then, the cause of MS has been a focus for many studies with numerous theories being put forward, many of which involves the effect of certain toxins or infectious agents. However, no single causative factor of this type has been unequivocally shown to induce MS. As an alternative explanation, in the 1930s Thomas Rivers showed that MS like disease was developed by injecting laboratory animals with myelin, thereby supporting the notion that MS might be an autoimmune disease where loss of tolerance lead to an attack by the own immune
system to myelin [49, 50]. What remains unknown in MS is the cause of the loss of tolerance, and viruses are still today considered a possible contributory factor, with special focus on Epstein Barr Virus [51]. Other suggested environmental triggers with support from larger studies are low vitamin-D levels and smoking [52].

The disease course of MS is generally classified into four different sub-types: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) and progressive relapsing MS (illustrated in figure 2). While RRMS is characterized by acute inflammatory attacks followed by periods of recovery, progression is associated by continuous loss of neurons and brain atrophy, but less signs of active inflammation [53]. Approximately 85% of patients initially develop a relapsing-remitting disease, but where a majority converts to secondary progression within 10-20 years. However, disease course is much dependent on age at onset (AAO). In late onset MS (AAO > 50), 83% of patients develops PPMS, while in young patients (AAO< 40) 94% initially develop RRMS and later convert to SPMS [54]. It could thereby be speculated if PPMS can be characterized as SPMS “amputed” from its relapsing remitting phase. RRMS and PPMS onset patients in average reach the same accumulated disability level at the same age, not after certain duration. In light of this fact it has been argued that MS possibly is a primary neurodegenerative disorder, and that inflammation is a secondary event to neurodegeneration [55].

**Figure 2:** Schematic view of different disease courses in MS. A relapsing remitting onset is most common, and the vast majority, but not all, later enters a progressive phase, secondary progression. There are also onsets with primary progression with or without acute attacks. Primary progressive onset is more common at later age of onset, and relapsing remitting at younger age.
However, current knowledge of MS genetics argues against the hypothesis of primary neurodegeneration. Recent advances in the genetic analysis of risk loci in large materials and GWAS point out a complex genetic predisposition, with a large number of genes contributing to a small increase in disease risk. A majority of the identified risk genes are involved in regulation of immune response. Recently, a large GWAS was conducted identifying over 50 risk genes, where only two could be associated with a neurodegenerative pathway in the absence of inflammation [56]. Of all identified risk genes, the HLA complex shows the by far strongest association and also associates in virtually all genetic studies [57]. The effect within the HLA complex has during the past years been studied in detail and the *HLA-DRB1* alleles have been defined as the strongest genetic risk allele. Overall, the effect of genetics on disease risk is strong, which was first noticed by evidence of familial aggregation with 300-fold increased risk for monozygotic twins and 20-40 fold for biological first degree relatives but with no increased risk in adoptees [58]. In spite of all effort with numerous GWAS studies, the accumulated risk of all susceptibility loci do not explain the observed heritability. This missing heritability could possibly to some extent be explained by gene environmental interactions. An example of this is smoking, where a recent study identified an interaction with HLA risk alleles. Compared to non-smoker without risk alleles, smokers without risk alleles had an odds ratio of 1.4, non-smokers with risk alleles 4.9 while smokers carrying risk alleles has an odds ratio of 13.5 [59].

Severity in MS is assessed by the Multiple Sclerosis Severity Score, (MSSS). This is a measure based on the Expanded Disability Status Scale (EDSS), a clinical measure of neurological deficit [60]. EDSS together with disease duration, counted from the first demyelinating symptom, generates a two dimensional matrix with MSSS values based on observations in a large MS material and the value predicts progression and development of neurological deficits over time [61]. However, it does not take into account age of onset that previously has been described to largely affect disease course. Further, the accuracy of the MSSS score during the first years of the disease is less accurate than later. Possibly, this can be due to a large inter-individual variation in noticing and remembering the first demyelinating symptom. There is still no established risk gene for severity, and the genetics of severity likely differs from susceptibility. Three GWAS have addressed severity and found a number of candidates [62-64], but none of them has been confirmed in independent materials.

There has recently been a fast development in new therapeutic strategies in MS. These mainly target the immune system and lower the relapse frequency in RRMS. However, still there is no effective drug for progressive MS. First line drugs for MS, mainly IFN-beta, do not have a significant effect on MSSS [61]. However, more recently a new treatment has been developed, natalizumab, that most effectively impedes the transmigration of lymphocytes into the CNS and that displays a significant effect on disease progression and MSSS in RRMS [65, 66]. The mechanism of action for
natalizumab also supports the nature of MS as an autoimmune disease. Longer follow-up studies on natalizumab treatment in the years to come can reveal if there is an effect also on conversion to progressive disease, i.e. if highly active immunomodulatory treatment during the inflammatory RRMS phase can diminish the risk of later conversion to SPMS. A number of different drugs with immunomodulatory actions are currently under testing, however virtually only for RRMS. Thus, the most important treatment question for the future will be to develop effective treatments also for progressive disease. However, a necessary requisite to do so is clarify more in detail the underlying molecular mechanisms operating in progressive disease.
4 OXIDATIVE STRESS AND NEURODEGENERATION

4.1 OXIDANTS AND ANTIOXIDANTS

Nearly all complex living organisms are dependent on oxygen for their survival. At the same time, oxygen is a highly reactive molecule that can cause damage to the organism as oxygen can form highly reactive chemical molecules, called reactive oxygen species (ROS). An oxidant, more often referred to as a free radical, is a molecule that lack one or more valence electrons and thereby attracts electrons from other molecules (since free electrons are almost non-existing in aqueous solutions). Therefore, there always needs to be a donor of the electron, the so called reductant. The electron transport reaction always includes these two molecules and the reactions are termed redox reactions.

In cell biology, the two main oxidants are ROS and reactive nitrogen species. ROS is constantly produced in cells as part of the electron transport chain in the mitochondria that generates energy for the cells. It further plays an important role in defence against pathogens and is induced by inflammation [67]. Low levels of ROS are also a part of cell signalling mechanisms. Cells have developed a defence system against oxidative reactions by generation of molecules that can scavenge radicals and thereby prevent cellular damage: antioxidants. Among these are uric acid, the vitamins C and E and glutathione. This reaction occurs spontaneously but cells have also developed enzymatic reactions to enhance the efficiency of the process. Oxidative stress occurs when the generation of oxidants exceeds the antioxidant capacity. It is therefore, especially in the context of the results and discussion of this thesis, worth to emphasize that oxidative stress and oxidative damage are dependent on the balance between generation of oxidants and the cells detoxifying capacity.

A main source of ROS in the CNS is from the respiratory burst system of activated microglia, which during inflammation release large quantities of ROS to the surrounding [68, 69].

4.2 LIPID PEROXIDATION

Free radicals can cause oxidative modification of lipids and initiate a process called lipid peroxidation (LPO). Polyunsaturated fatty acids (PUFA) are most easily oxidized due to the easily broken carbon double bonds. Therefore, saturated or monounsaturated fatty acids are less sensitive to oxidation. In the initiation step, a hydrogen atom is attracted from a lipid (LH) by a free radical, which generates a lipid radical (L●). This undergoes molecular rearrangements to form a conjugated diene which easily reacts
with oxygen, forming a lipid peroxy radical (LOO●). This lipid hydroperoxide easily attracts a hydrogen atom from an adjacent lipid generating a lipid hydroperoxide (LOOH) and another lipid radical (L●). This way, one initiating event can lead to hundreds of modified lipids until the reaction is terminated (figure 3).

Figure 3: Lipid peroxidation is a chain reaction, where one oxidized lipid generates another until the reaction is terminated. A single initiating event by a radical can thereby destroy hundreds of lipids and generate large amounts of reactive aldehyde break down products if not rapidly terminated. The amount of damage by reactive aldehydes in tissue will depend on variation at three different levels: Number of events at initiation, which is determined by production and detoxification of ROS; Degree of propagation where ability to terminate the initiated chain reactions is limiting and finally; Capacity to efficiently detoxify the produced aldehydes. Gsta4, which is the focus of this thesis, is involved in the last step, detoxification of the produced aldehydes, especially HNE, thereby preventing these from reacting with other molecules in the surrounding tissue.

Since lipid peroxidation is a chain reaction it can, if not rapidly inhibited, easily destroy lipid rich areas such as cell membranes or myelin sheaths. In addition to destruction of lipid layers, cleavage of the fatty acid carbon chains of the lipid hydroperoxides results in generation of highly reactive aldehydes such as malondialdehyde (MDA), acrolein and 4-Hydroxynonenal (HNE). These have, compared to ROS, a relatively long half-
life (HNE up to 2 minutes in tissue) and can therefore diffuse to sites distant to the
initial oxidative event and there react to other molecules such as proteins or DNA [70].
HNE, which is in the focus for the results in this thesis, is a 9 carbon molecule with
three reactive sites generated by cleavage of ω6 PUFAs and mainly reacts with the
amino acids cysteine, histidine and lysine. The detailed chemical reactions for HNE
generation have been reviewed elsewhere [70, 71]. Although, most studies on HNE
focus on toxicity, it should be noticed that it also acts as an important signalling
molecule at subcytotoxic concentrations [72].

The CNS is particularly vulnerable to lipid peroxidation due to its high content of fat
and particularly PUFAs. The most common PUFA in the brain is the ω3
Dexosahexaenoic acid (DHA) followed the ω6 Arachidonic acid (ARA) [73]. Myelin,
which surrounds the axons and is the target of the immune attacks in MS consists of
30% protein and 70% lipids.

Increased age is associated with more lipid peroxidation and suggested to be an
important mechanism in normal aging. [74, 75]. An interesting observation is that
animals with a longer life span seems to have lower degree of unsaturated and more
saturated fatty acids in membranes [76, 77]. A fatty acid composition that is more
resistant to peroxidation correlates with longevity [78-80]. Studies of C. Elegans
transfected with mGsta4 and other HNE metabolizing GSTs show a direct correlation
with HNE conjugating activity and lifespan [81]. Gender is possibly an important
parameter to take into account in neuronal damage of lipid peroxidation. Males have
been shown to have higher levels of lipid peroxidation markers after severe TBI [82],
and this has been proposed to possibly be an effect of the antioxidant effects of
oestrogen [83].

4.3 GLUTATHIONE AND GLUTATHIONE S-TRANSFERASES
The first enzymatic defence process against oxidative stress in cells is superoxide
dismutase, SOD, that catalyses the reaction of two superoxide molecules into oxygen
and hydrogen peroxide (H₂O₂). The second step, to metabolize H₂O₂, uses the enzyme
catalase or peroxidases. Among the peroxidases are glutathione peroxidases (GPxs),
which reduces H₂O₂ to water by conjugation to glutathione.

Glutathione is a tripeptide made up of glutamate-cysteine-glycine (Glu-Cys-Gly). This
antioxidant is abundant in high concentrations in all cells. Most of the glutathione
within normal cells exist in reduced form (GSH) and only a small part in an oxidized
state (GSSH)[84]. An increased GSSH/GSH ratio is a sign of oxidative stress. Together
with the thioredoxin system, glutathione system is considered to be the major redox
regulating system in mammalian cells.
Glutathione S-Transferases (GSTs) constitute a large family of proteins that are divided into different classes based on their structure, but also substrate selectivity [85]. There are seven classes of mammalian cytosolic GSTs described, Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta, that generally share >40% identity within and <25% between subclasses [86]. Other classes are also described in non-mammalian organisms. GSTs are highly abundant in most mammalian cells and can compose up to 10% of the cytosolic proteins in some organs [87].

Glutathione plays an important role for the defence against lipid peroxidation by terminating the chain reaction. This reducing conjunction is known to be catalysed by glutathione peroxidases (GPxs), which catalyse the reduction of lipid hydroperoxides, but also by various GSTs. GSTs also catalyse the reduction of electrophilic by products of lipid peroxidation, such as HNE and acrolein, by conjugation to GSH thereby preventing them from harmful reactions to other cellular molecules [88].

The glutathione pathway is the main cellular defence system for reducing HNE, by formation of stable glutathione-HNE adducts (GS-HNE), although other routes are also present [72]. HNE can spontaneously react to the highly abundant GSH molecules through so called Michael addition. However, the reaction is also catalysed by GSTs with an activity rate 600 times faster than the spontaneous reaction [89]. The main HNE reducing enzyme is Gsta4-4, which in mouse studies is indicated to make up approximately 70% of the reducing capacity in the brain [90]. Gsta4-4 seems to have evolved to reduce HNE not only by high catalytic activity, but also by having an exceptional resistance to HNE adduction itself [91]. Mammalian GSTs are dimeric proteins and named according to their subunit composition [92] The dimeric protein product of the gene Gsta4 is thereby named Gsta4-4.

4.4 LIPOID PEROXIDATION IN NEURODEGENERATIVE AND AUTOIMMUNE DISORDERS

Oxidative stress and lipid peroxidation has been implicated as a pathogenic process of neurodegenerative disorders and accordingly the focus of numerous studies. The list of examples is long and there are also numerous reviews on the subject, for example [93, 94]. A few examples worth to mention in relation to the data presented here are that HNE is considered a marker for lipid peroxidation, and has been detected in elevated levels in CSF of ALS [95], PD [96] and AD [97]. Further immunohistochemical analysis show presence of HNE in PD [98], AD [99], ALS [100] as well as in lesions of MS [101].

Oxidative stress and LPO are possibly not only a consequence of disease and a mediator of progression, but possibly also a contributory factor to disease development.
SOD1 variants are the main risk allele in ALS indicating the importance of oxidative defence status for disease development. In terms of extreme exercise being a risk factor for ALS it is worth to notice that studies show that high-intensity exercise induce oxidative stress and lipid peroxidation [102, 103], while moderate exercise can be regarded as an antioxidant [104].

In AD, oxidative damage is an early event in the disease course. Highest levels are seen initially and actually decrease with disease progression, also implying importance for disease development [105]. The amyloid beta peptide Aβ(1-42), found in the senile plaques of AD, has oxidative properties and is known to induce lipid peroxidation and formation of HNE [106]. Further, long term exposure to pesticides that induce oxidative stress is proposed to be involved in pathogenesis of PD including earlier onset and these are also used to induce disease in experimental models [107, 108].

Oxidative stress and lipid peroxidation is also considered a major mechanism contributing to secondary degeneration after TBI [109-113], where reactive by-products of LPO is suggested to play a role [114]. HNE levels are elevated after TBI with a correlation to age in disease models [115]. Various antioxidant treatments have been suggested from animal experiments but so far failed to show effect on outcome in clinical trials in TBI [113].

Furthermore, oxidative modification of proteins has been implicated as a possible cause of loss of tolerance and development of autoimmune responses [116]. Smoking is a major risk factor in immune mediated disorder, extensively reported in RA [117-119] but also SLE [120, 121] and MS [59, 121]. Cigarette smoke is known to induce oxidative modifications to proteins and lipids [122]. Likely, increased immunogenicity from protein modifications by smoke is at least a contributing mechanism among many factors for increased risk of autoimmune disease, a notion that is supported by gene-environment interaction of HLA and smoking [59].

In MS, smoking is not only a risk factor for disease but also seen to be associated with more lesions and a shorter time to transition into progressive disease phase [123]. In terms of MS, an autoimmune disorder with a prominent neurodegenerative facet, reactive aldehydes can play a role both as neurotoxins and inducers of a stronger autoimmune response. MDA-modification of MOG has in EAE been shown to induce a more aggressive disease course than unmodified MOG [124]. An interesting recent finding in MS with relevance to smoking, is presence of the same oxidized phospholipids in lesions as has been associated with inflammatory disease of the lung, with presence of antibodies against these in CSF and lesions [125]. Specificity of the OCB has been an intense focus for studies during 40 years since their discovery. However, recently, with the use of lipid arrays, it was reported that around 30% of the intrathecal antibody response is directed towards lipid epitopes [126]. Perhaps,
initiating events to break tolerance can occur outside the CNS, as in the lung, and later lead to activation of an intrathecal autoimmune response.

Systemic Lupus Erythematosus (SLE) is a disorder characterized by presence of anti-nuclear autoantibodies (ANA). The most common is against double stranded DNA (dsDNA) which occurs in approximately 70-80% of the patients [127]. Protein modification has been shown to be a major cause of autoantibody generation in SLE [128]. Approximately 40% of SLE patients have autoantibodies against the 60KDa-Ro protein [127]. Studies in a model of SLE has shown that HNE-modified Ro results in a stronger autoimmune response and also development of so called epitope spreading [129]. This is a progression mechanism where autoantibodies towards other epitopes than the initial immunogen are developed. In mice, presence of anti-dsDNA antibodies is found when immunized with HNE modified Ro but not after immunization with unmodified protein [130]. Further, 30-40% show antiphospholipid antibodies [131], which is associated with increased lipid peroxidation and epitopes of oxidized phospholipids [132, 133].

4.5 GSTA4 IN NEURODEGENERATIVE AND OTHER DISORDERS

A possible role of Gsta4 in neurodegenerative disorders has mainly been studied in Parkinson’s disease. Geographic distributions of the disease has for a long time pointed towards important contribution of environmental factors and particularly to exposure of pesticides, and disease models have been developed with induction by pesticides [134]. The two pesticides, Maneb and Paraquat, has in combination been shown to have an effect on Gsta4 expression after long term exposure [135]. Low dose ip-injection of Paraquat significantly has a stronger life shortening effect in Gsta4 knock-out mice compared to controls [90]. Pesticides are known to induce oxidative stress and GST activity [136]. A small association study between Gsta4 and Parkinson failed to show any association, but only included 60 PD and 60 Healthy controls [137].

Further, SOD1 knockout mice, used as a model of ALS, show a normal development up to a certain age. Expression profiling has revealed that these young mice have one particularly strongly up regulated gene, Gsta4, likely as a compensatory mechanism [138]. In the same way, Gsta4 knock-out mice activates compensatory mechanisms [139, 140], indicating a complex regulation of total antioxidant capacity.

Higher expression of Gsta4 has been noted in key brain areas for alcohol prediction in alcohol preferring rats, which also show a longer expected life span [141]. A common link is the enzyme aldehyde dehydrogenase, which is as important enzyme in breakdown of alcohol but that also exerts a substantial catalytic activity against HNE.
Double knock-out mice for Aldh1 and Aldh2, present in dopamine neurons, show age dependent motor deficits, and increased HNE levels [144].

A recent study with a forward genetic approach very similar to the studies of this thesis, pinpoint Gsta4 as a susceptibility gene for non-melanoma skin cancer [145]. This mouse study also uses global expression gene profiling together with congenic strains, and identify expression differences in Gsta4 as underlying the difference in melanoma susceptibility. Genetic variation is here also associated to human case/control data set. Gsta4 has also been shown to be strongly up regulated in keratinocytes upon UVB irradiation as a defence against oxidative stress [146, 147].

Finally, there are several in vitro studies showing that overexpression of Gsta4 protects cells from oxidative and HNE induced injury [148, 149]. Altogether, previous published data points towards Gsta4 activity as an important parameter for cell survival under conditions of oxidative stress. The forward genetics approach in this thesis, pinpointing Gsta4 levels, as a main determinant of differential nerve cell survival between rat strains further strengthens this notion. Further studies, both genetic and mechanistic, will reveal if there is common variation in Gsta4 activity that is of pathogenic importance in human neurodegenerative disorders.
5 METHODOLOGY

5.1 EXPERIMENTAL MODELS

Disease models have been developed for a number of disorders and enable both genetic and mechanistic studies that are not possible to conduct in human disease. A main advantage is the possibility to reduce sources of variation by standardized induction protocols, control environmental factors and exclusion of factors that are not part of the hypothesis. Further, disease models enable, at least in theory, unlimited sample size under these conditions.

All animal models have been performed in a standardized manner under deep anaesthesia with isoflurane.

5.1.1 VRA, Ventral Root Avulsion

Ventral root avulsion is a nerve injury model where lumbar ventral roots, containing axons from motoneurons within the ventral horn, are detached on the border between the CNS [150]. This results in activation of a local inflammatory response, with activation of microglia and astrocytes, infiltration of T-cells and a retrograde degeneration of motoneurons. Neuronal death starts to be detectable approximately one week after injury and most motoneurons dies during the second and third postoperative weeks [151]. Two of the main advantages with this model are that it is highly reproducible with a low degree of inter-individual variation and that it causes a minimal damage to the blood brain barrier, thereby mainly affecting local reactions within the CNS.

Figure 4: Schematic overview of VRA. Ventral roots L3-L5 are avulsed leading to degeneration of motoneurons in the ipsi-lateral quadrant, activation of glia and formation of scar tissue. The micrograph to the right depicts a Cresyl violet staining from a DA rat 21 days post injury with a substantial neuronal loss in the ipsilateral side. The picture further shows the piece of the spinal cord cut out and used for expression analysis with removal of scar tissue.
After VRA, spinal cord has been collected and further analysed for disease markers at different time points after injury. For neuronal counts, spinal cord has been collected 21 days post injury when number of surviving motoneurons has stabilized. Neurons have been stained with Cresyl Violet and relative numbers achieved by counts on the injured side using the contralateral side as internal control. Sections have also been used for immunohistochemical analysis with antibodies. In general, two segments (L4-L5) have been used for histology. Tissue has also been collected for expression studies 5 days post injury, and then mostly the L3 segment. In this case, only the ipsilateral quadrant of the segment has been saved with careful removal of scar tissue (Figure 4).

5.1.2 TBI, Traumatic Brain Injury
The variation in traumatic injuries is extremely large where no injury is exactly similar to another. A model is therefore very valuable for studies of genetic impact on the outcome. There are many models for different types of TBI such as weight drop, controlled cortical and fluid percussion injury for contusions, a model for diffuse axonal injury, blast injury etc. In the model used in this thesis, we have induced contusion with a weight drop model [152]. In this model a craniotomy is performed 2.3 mm lateral and 3 mm behind the bregma with great care not to damage the dura. A rod is placed on the exposed dura and a 24gr weight is allowed to fall on the rod from 70 mm height and compress the tissue 3mm. In this way a standardized parietal contusion is achieved while the animals do not present any discernible clinical symptoms. The weight drop injury model has been extensively studied with regards to pathophysiological processes after TBI such as inflammation, oxidative stress and apoptosis [153-155].

The search for biomarkers in the serum or CSF of patients with TBI could serve both as a diagnostic and prognostic tool and also help us monitor a specific pathophysiological mechanism. Indeed, serum and CSF S100b, an astrocytic protein, has prognostic value in human TBI and serves as a severity marker. With regards to our congenic and Gsta4, which detoxifies HNE we wanted therefore to measure markers for neuronal injury and ideally HNE adducts in the CSF of animal TBI. We therefore obtained CSF from rats after TBI. For this purpose, a technique to collect CSF post mortem in the rat was established, where a 23G butterfly needle is used and the plastic cover is cut to expose 3-4 mm of the needle tip. A stereotaxic frame is used and the rat is shaved over the occipital crest, where after the needle is inserted into the medullary cistern and approximately 100uL of CSF is drawn. Only clear CSF, free from sign of blood contamination has been used for analysis.
5.1.3 EAE, Experimental Autoimmune Encephalomyelitis

EAE is a model of MS where an autoimmune reaction, with demyelinating lesions, is caused by immunization with CNS myelin antigens in combination with adjuvants. The CNS antigens can be composed of whole spinal cord homogenates, myelin or specific myelin proteins. Disease could also be elicited with transfer of myelin specific T-cells [156]. With the use of different antigens, immunization protocols and genetic background, varying features and types of MS can be mimicked.

In paper IV of this thesis EAE has been induced with myelin oligodendrocyte glycoprotein (MOG), a small protein exposed on the surface of the myelin sheets. This result in a disease onset 10-14 days post-immunization and a relapsing-remitting disease type with many characteristics similar to MS disease course [157]. The rats are scored daily for clinical symptoms up to maximum 60 days post injury. Also here, the same technique to take CSF as applied in the TBI model has been used. Various CSF parameters are important diagnostic tools in MS and CSF from EAE rats therefore enable evaluation of known MS markers. Although MOG-EAE is considered to be the best model for MS it still has clear differences to human disease. One important, with respect to the data presented here, is that it is an acute disease which is followed only for approximately one month and therefore better reflects the molecular mechanisms of an acute inflammatory attack/relapse rather than the long term progression of MS.

5.2 GENETIC ANALYSIS IN LABORATORY ANIMALS

Genetic studies in laboratory animals make use of inbred strains, which are homozygotes at each locus in the genome. A strain is considered inbred after at least 20 generations of brother/sister mating. Around 1910 inbreeding of rat and mouse strains was initiated in different labs and many of these early strains are now among the most widespread in laboratories around the world, for example the Wistar rat and the C57 family of mouse strains. By the use of more than one inbred strain with different response to the model, average outcome values of groups can be obtained in order to evaluate pure genetic effect without other influences. This is referred to as the relationship between genotype and phenotype, and the terms were first defined by the famous Danish botanist Wilhelm Johansen from his studies of beans 1903 [158].

5.2.1 Inbred rat strains

In the studies presented here two different rat strains and crosses of these are described, Dark Agouti (DA) and Piebald Virol Glaxo (PVG). DA is widely used in immunological research, since it is susceptible to autoimmune diseases such as arthritis and EAE. DA is also highly susceptible in VRA, where it has a high degree of neuronal cell death, stronger activation of glial cells with high expression of MHC class II and high degree of infiltrating T-cells. PVG, in contrast, is more resistant to EAE (although
it is a dose threshold effect) and arthritis, and shows lower local inflammatory response and better nerve cell survival after VRA. PVG is somewhat larger than DA, but the phenotypic effects on the above mentioned parameters are not dependent on size.

5.2.2 Crosses
Crossing strains result in recombination between chromosomes and thereby random fragmentation of the inbred strains chromosomes. Crossing two strains creates an F1 generation where all individuals are identical and heterozygotes at all loci. However, subsequent crossing of the F1 generation will result in an F2 intercross where each individual has a unique combination of alleles from the donor strains. On average, at each locus, 25% will be homozygote for strain A alleles, 50% heterozygotes and 25% homozygotes for allele B. Since recombinations only occur at 1-2 places on each chromosome, the fragments are large. Continuous crossing of F2 individuals with controlled random mating of at least 100 individuals will create offspring that accumulate more and more recombinations for each generation. This type of cross, termed advanced intercross line (AIL), thereby give a better resolution in genetic mapping [159]. An alternative approach is to backcross the F1 animals with one of the initial inbreds, creating only individuals homozygote for the backcrossed strain and heterozygotes. Backcrosses (BC) are particularly useful for studying so called heterosis effects, where heterozygotes display the strongest phenotype. A schematic view of breeding strategies is depicted below in figure 5.

5.2.3 Congenic strains
In congenic strains, a size limited fragment from one strain is introgressed on the genetic background of the other strain. As in a BC, a F1 generation animal is backcrossed to the strain that should be the background strain. The progeny is then genotyped and an animal with the desired genetic fragment is backcrossed again to the background strain. This is continued for at least 10 generations after which only approximately 0.1% of background is left, since only half of the background on average will be transferred for each generation (0.5<0.001). An alternative is the speed-congenic approach, where the background is genotyped in each generation. On average 50% background genome will persist, but follow a normal distribution with individuals displaying between 30 and 70% background. Offspring with lowest level of background but with preservation of the desired fragment is selected for further backcrossing [160]. By this approach fewer generations are required to obtain the same low level of background as for traditionally bred congensics, but it is more labour intense in terms of genotyping effort.
Figure 5: Breeding scheme for different mapping approaches as described in the text. Two different strains are crossed and generate an F1 generation. Another generation of intercrossing of F1 individuals create an F2 that further can be intercrossed to generate more recombination in an AIL. F1 generation can also be backcrossed to either of the two initial strains to generate a BC. Further backcrossing with positive selection for genomic region of interest creates a congenic line.

In contrast to knock out strains that completely lack expression of a gene, congenic animals in most cases better reflect human disease, where total absence only occur in rare cases but variation in expression levels and function is more common. However, a drawback with the congenic approach is that it is rare to get down to single gene congenics. This requires very large sample size of backcrossing and definitely a fair bit of good luck. The result is that the fragment often contains at least a handful of genes that all possibly could affect the studied phenotype.

5.3 Analysis of Genotype versus Phenotype

A genetic marker is a common variation in the genome that differs between individuals. The rat studies have used microsatellite markers, which is tandem repeats of 2-6 base pairs. By polymerase chain reaction, PCR, the genomic fragment of interest is amplified and the size of the amplified fragment is determined by electrophoresis. The human data make use of single nucleotide polymorphisms, SNPs. These are single base pairs in the genetic code A/T/G/C that are substituted to another base. Genetic markers
occur frequently in the genome with microsatellite markers approximately 1 in 14 000bp [161] and SNPs 1 for every 900bp [162]. SNPs thereby enable extremely dense genetic maps.

There are two main approaches in identifying risk genes, linkage and association, that are described further below. In animal studies, identification of a single candidate gene mostly relies on multiple strategies such as linkage, overlapping congenic strains and expression analysis (figure 6).

5.3.1 Linkage analysis

In linkage analysis, families are studied over generations in order to find genetic markers that co-segregate with the studied trait within the family. Thereby certain parts of the genome can be linked to the observed trait. Since all individuals within a study have to be related the number of samples in the studies is limited. Given the limited sample number, linkage analysis requires mutations with high penetrance. Therefore, this methodology has mainly been successful in finding monogenic mendelian traits. Examples of successful linkage analysis are Huntington’s disease [163], cystic fibrosis [164, 165] and breast cancer [166], but there are also examples in complex disease such as APOE in AD [167, 168].

Finding risk genes in experimental populations generally start with linkage analysis in an F2 intercross. Since the inbred rats are identical in their genome, a theoretically unlimited sized family can be created already in the second generation, largely improving the power to detect gene regions. Larger sample size will enhance the chance to detect traits with lower penetrance and also increase the total amount of recombination, enabling detection of a narrower region. An identified gene region that regulates the studied phenotype is called a quantitative trait locus, QTL. The VRAI QTL regulating neurodegeneration after VRA that has been investigated here was initially detected in an F2 intercross between DA and PVG [169] with interval mapping. Interval mapping was first described by Lander and Botstein in 1989 [170] and is a principle where multiple markers are analysed at the same time and the genotypes between markers are simulated based on recombination frequencies.

The result of the linkage analysis is the likelihood that the trait pattern is linked to the genotype at the genomic position. This is often presented as the logarithm of odds, LOD. This score represents the likelihood of presence of a QTL versus the likelihood of no QTL, as a logarithm with the base of 10. This implies that with a LOD score of 3, the presence of a QTL is 1000 times more likely than no QTL. Likelihood ratio score (LRS) is also sometimes also used and is directly convertible to LOD, where LOD = LRS/4.61. In general, LOD scores above 3.3 are considered to be of significance in experimental crosses [171]. Empirical significance levels for a certain phenotype can
also be calculated by permutation tests where the data is randomized and analysed multiple times (generally at least 10 000) and X% significance level corresponds to a genome wide lower LOD score in 100-X out of 100 simulations [172].

In order to establish the genomic size of the QTL a confidence interval (CI) is determined. There are several different methods to establish the length and commonly used is the LOD drop method. A LOD drop of 1.5 is generally considered to correspond to a 95% confidence interval but sometimes, especially in AILs, the stricter 1.8 is used [173, 174]. This has been used in paper one together with linkage analysis with the software R/QTL [175].

Figure 6: Principle of identifying candidate genes in experimental population. Solid line depicts LOD score from an F2 intercross with large CI. Dashed line shows how the fragment is reduced with an AIL. Further, overlapping congenic fragments (white fragments with phenotypic effect, dashed without) further reduce the interval obtained by the AIL to a more narrow region.

5.3.2 eQTL mapping

In paper II we make use of expression QTL (eQTL) mapping to detect candidate genes. An expression QTL is basically a region that regulates gene expression, rather than disease phenotype. However, eQTLs and a disease QTL that co-localize to the same marker can possibly link disease to expression pattern [176].

The rapid development of chip based screening techniques in the last decade has enabled the possibility to study the whole transcriptome in a tissue, so called global gene expression profiling. By combining global expression profiling with linkage analysis, all expressed genes are checked for linkage to all genotyped markers in the genome. This generates a two dimensional matrix with data of transcripts mapping to any or multiple locations in the genome.

eQTLs are divided into cis- or trans-acting QTLs. A cis-acting QTL is defined as expression regulated from the same position as the gene is located in the genome. This could from example be explained by differences in promoter sequence. A trans-
regulated QTL maps to a position distant to the location of the gene. A large group of trans-regulated transcripts at a marker, trans-cluster, could for example be explained by differences in a transcription factor. Extremely generalized, one can state that a cis acting QTL is likely to be the causative gene, while trans-regulated transcripts are likely to be a consequence of the cis-regulated gene and can give important information about function of the cis-regulated gene. Of course in reality it is often more complex and more factors need to be taken into account that however will not be discussed further here.

5.3.3 Association study
The second approach is association, where groups of individuals are not genetically related is evaluated. Simply, allele frequencies are compared between two groups and if an allele is significantly more common in one group, this variant is considered to be associated with the trait. Association studies are generally more successful than linkage studies at finding genes variants with low penetrance, as most often is the case in complex disease. Since very large sample cohorts can be collected, which are not achievable in family studies, it becomes possible to detect a small overrepresentation in a group with statistical significance.

The last decade, with the development of better genotyping techniques there has been an increasing number of large scans in various complex diseases where SNPs across the whole genome are scanned for a particular trait, so called genome wide association studies (GWAS). These are most often case/control studies where large sample sizes are used that enables detection of risk genes that contributes with a small increase in risk of developing a certain disease. The advantage of these studies is that this is an unbiased approach in search for genes without the use of previous knowledge that enables detection of new genes and mechanisms not previously associated to a certain disease. However, this also results in a large number of independent tests which raise the threshold level for significance a lot because of correction for multiple testing. In addition, GWAS studies have mostly been conducted with the hypothesis that a common disease also is caused by a common gene variant. Therefore, the chips used for SNP typing almost entirely include SNPs with a fairly high minor allele frequency. Last, it should also be noticed that GWAS rarely have analysed haplotypes and all these factors can possibly lead to that existing associations have not been detected.

A large number of risk genes, all with a small contribution to the overall risk, have today been detected in various complex disorders with numerous GWAS. However, the sum of all these risk factors does not by far reach the observed heritability seen in familial studies. This discrepancy is often referred to as the missing heritability and a number of sources have been proposed [177]. One is large contribution of many rare gene variants in the population. Common gene variants, used in association, are
variations with long history which are widespread in the population. However, in each
generation an estimated 175 new gene variants arise, where different rare variants can
have a large effect at individual level on a common disease, but cannot be detected in
association studies because of lack of linkage disequilibrium (LD) [178]. Another is
presence of gene-gene or gene-environmental interactions that, as discussed in the
introduction of the threshold model, means that total risk is not additive to individual
risk factors but rather multiplicative. A third reason could also be over estimation of the
heritability in the first place [179].

In paper IV, an association study to MS for GSTA4 gene variants has been conducted
with 9 SNPs covering the genetic variation in the GSTA4 gene. Coverage is calculated
based on LD for SNPs generated in large reference materials which are available in the
HapMap project for various populations. Frequencies from the Central European
(CEU) population group have been used for this project in order to represent the
analysed material. If two SNPS show high degree of LD, this implies that their
distribution differs from what can be expected by chance because these are inherited
together. This is referred to as tagging SNPs, where a tagging SNP can be expected to
reflect the genotype also for many other SNPs in the region.
6 RESULTS AND DISCUSSION

6.1 ISOLATION OF A SMALL GENETIC FRAGMENT AFFECTING NEURODEGENERATION (PAPER I)

Previous work on neurodegeneration in different inbred rat strains had among the tested strains identified DA and PVG as displaying the least and highest percentage of surviving motoneurons after VRA, with 26% and 50% survival, respectively [151]. To identify possible regulatory regions for this trait a F2 intercross between DAxPVG had been set up with 186 rats and phenotyped for inflammatory markers and neurodegeneration [169]. This study identified two gene regions affecting neurodegeneration; VRA1 on chromosome 8 with a significant linkage peak and a less strong effect on chromosome 5, VRA2, with a suggestive linkage.

In paper I, we initially introgressed a genetic fragment with the size of the confidence interval of VRA1 from the less susceptible strain PVG onto DA background. Further this fragment was split up into the three recombinants R1, R2 and R3, covering different parts of the interval. The congenic rats were bred with traditional approach and with extensive recombinant breeding the number of backcrosses widely exceeds the minimum level of 10 generations. The three recombinant strains were tested for neuronal survival and, indeed, the protective effect previously seen in the F2 was reproduced in the congenic strains R1 and R3 but not R2. This reduced the VRA1 QTL to 9 Mb with 45 protein coding genes located in the smaller R3 fragment. In addition, a reciprocal congenic with susceptible fragment on resistant background was bred. This strain displayed significantly lower survival rate compared to the resistant PVG strain, but still better than the susceptible DA strain. Altogether, this confirms the findings from previous F2 intercross of gene region with a significant effect on nerve cell survival, but does not identify the exact gene.

Subsequently, two advanced intercross lines (AIL) of 8th and 10th generations, previously used for fine mapping of a MHC class II effect [180], were used to reproduce and fine map the linkage. The genetic effect of VRA1 was reproduced in both AILs, but only just above significance level obtained by permutation test. In general, the better genetic resolution in an AIL has the cost of lower power [181], which implies that a somewhat larger population size is needed. Therefore, a combined analysis of both crosses was conducted which increased the linkage peak significantly and thereby also reduced the confidence interval. However, the confidence interval did not reduce the interval further than the smallest congenic, R3, and thereby did not decrease the number of candidate genes, but nevertheless gives further evidence for the importance of the genetic region by providing independent replication.
In addition, the suggestive QTL VRA2 was also analysed in the AILs. This QTL was reproduced in the larger G10 material, but not in G8. However, a multiple QTL model in G10 for VRA1 and VRA2 reveals a highly significant additive effect of both QTL. No such results were obtained by similar analysis in G8. It is likely that the penetrance of the VRA2 locus is smaller than for VRA1 and thereby too low to be significantly linked with a population of the given size. However, the effect was not present at any level in the G8 cohort and a combined cross analysis did not increase the significance. Another possibility is also a more complex pathway that requires more factors of closely related genes that might not be inherited together as often in an AIL as in an F2. A two dimensional scan could not detect any significant interaction, but it cannot be excluded this is due to low power. Neuronal counts in the crosses are also performed at day 14, as a compromise to enable studies of both neurodegeneration and inflammation in the same material. A later timer point, where the difference between the strains is stronger would perhaps also have increased the significance in the genetic mapping.

With these data I conclude that VRA1 indeed is a region with high significance for nerve cell survival after injury and that VRA2 still is regarded as a region of suggestive importance.

6.2 GETTING DOWN TO SINGLE GENE LEVEL, GSTA4 AS A LIKELY CANDIDATE (PAPER II AND III)

The ultimate proof that a single gene underlies a trait is to demonstrate an effect in a single gene congenic. However, it is extremely rare to get down to single gene level and most often there is a set of genes were the likely candidate has to be identified by a series of indications pointing towards the mechanism. In paper II, we present a recombinant of the R3 strain, denoted R5, which reduce the previously reported fragment with an additional 3 Mb, down to 6 Mb and containing 35 protein coding genes.

In an effort to find genes underlying various traits studied in the VRA model an F2 intercross between DA and PVG was set up and material collected 5 days post injury for global expression gene profiling. This is approximately the time point just prior to when the motoneurons start to degenerate [182]. The time point was chosen to capture molecular events that determine the fate of nerve cells. At the later time points, 14 days, used in previous intercrosses most of the neurons are already gone, which may distort the molecular pattern underlying the variation. For expression profiling, the L4 segment was used and mRNA extracted to use with the Affymetrix Rat Gene 1.0 ST Array which contains more than 27 000 gene-level probe sets.
eQTL mapping, where all expressed transcripts are mapped to all genotyped markers in the genome, revealed only one strong cis-regulated gene within the R5 congenic fragment: Gsta4. In general, cis-regulated genes are likely candidate genes for a trait, while all trans-regulated transcripts might give important insights into mechanisms affected by the QTL and underlying genetic variation. There are four other cis-regulated genes on the two markers analysed in VRA1. However, all displaying much lower p-values and none actually residing within the R5 fragment.

Among the trans-regulated transcripts found in VRA1, many are genes with a link to neurodegeneration. Among the most striking is probably Dao, D-amino acid oxidase, with lower expression in the DA homozygotes, recently associated to familial ALS and the pathogenesis of motoneuron death in both mutant Dao and mSOD1 mice [183, 184]. In terms of oxidative stress, the trans-regulated transcript Duox1 is also of interest, since it is one of the NOX-enzymes, highly specialized in generating H₂O₂ on the cell surface. [185]. It is known to be induced by pro-inflammatory molecules and supposed to play an important role in various states of chronic inflammation [67].

Other genes with strong trans-regulation are Tap2, involved in antigen presentation and associated with SLE [186]. Ccl2, a pro-inflammatory chemokine reported to have dual effects with regard to neurodegeneration [187-189], Ucn, a protective neuropeptide that function to regulate stress response [190, 191], Tmod2 that is highly expressed in neurons and involved in synaptic plasticity [192] and Cyp39a1 involved in cholesterol metabolism with link to neurodegeneration [193]. A gene that possibly could be related to Gsta4 is Gclc, which is located as the first gene in the R3 fragment. The protein is involved in a rate limiting step of glutathione synthesis, and regulated by feedback regulation based on glutathione levels [194]. However, Gclc does not seem to map to the VRA1 region.

A custom made antibody shows that Gsta4 protein in the CNS is mainly located within neurons. No commercial antibody towards rat Gsta4 was available, as was data on cellular localization of Gsta4 in CNS. Rat studies dating 20 years back indicated that Gsta4 was mainly located in astrocytes and blood vessels [195], while human stainings have been reported for blood vessels and neurons [196]. However, here we provide data clearly showing that Gsta4 mainly is a neuronal protein within the CNS (Figure 7). This data will also be of importance for interpretation of other rat studies on Gsta4.
Figure 7: Micrographs of the ventral horn in rat stained with anti Gsta4 (red) and NeuN (green). NeuN is a neuronal marker and the mid picture clearly depicts the large motoneurons located in the ventral horn. Gsta4 staining is strong in the motoneurons, but also present in other neurons. In paper IV, micrographs with higher magnification of motoneurons are shown.

To further investigate the role of *Gsta4* in neurodegeneration, the R5 congenic was tested in a in the TBI model. Importantly, global expression profiling in DA and PVG after TBI shows that glutathione metabolism is the strongest regulated pathway between the strains. The results from TBI clearly showed that *Gsta4* has an effect on neuronal survival also after TBI. As after VRA, the R5 strain has a substantially better neuronal survival after TBI. Consistent with expression levels in spinal cord, brain tissue show similar levels of Gsta4 expression in R5 and PVG, but substantially lower levels in DA. Both in spinal cord and brain tissue, western blot analysis showed that expression differences translate into protein levels. This is important since it is the protein levels in the cells that affect enzymatic activity, not mRNA. This shows that the mechanism is of importance also for neuronal survival in trauma and therefore likely represents a general mechanism of importance for acute nerve injury.

Further, injection of HNE directly into the brain showed neurons that positively stain for HNE and a stronger up-regulation of *Gsta4* in R5 compared to DA. In particular, CA1 neurons of the hippocampus (located just under the site of injection) showed a strong up-regulation of *Gsta4*. Interestingly, these neurons are sensitive to delayed neuronal death in a model of cerebral ischemia, where they also stain HNE-positive [197]. We also showed co-staining of HNE and Tunel-positive nuclei indicating an in vivo role for HNE in apoptosis. In addition, levels of neurofilament light, a CSF marker for axonal injury, was also lower in the congenic compared to DA after HNE injection. Together, data point towards *Gsta4* variation as being a potential mechanism of general relevance for neuronal survival resilience in a variety of acute conditions.
6.3 THE MECHANISM IN HUMAN DISEASE, RELEVANCE FOR DISEASE SEVERITY IN MS? (PAPER IV)

Given the data from preceding papers, possibly GSTA4 could be of importance also for neurodegeneration occurring in MS. As mentioned earlier, MS is primarily an autoimmune disease, however, with neurodegeneration as a feature of high importance for disease severity and disability. Since GSTA4 in the CNS is mainly expressed by and located in neurons, expression or protein levels in human samples has not been possible to study.

First, we here conducted three different EAE experiments with DA and the congenic R5 strain. The first experiment, with milder induction protocol, showed a tendency towards lower disease activity in the congenic. Given the rather low number of individuals the difference was not statistically significant. Experiments 2 and 3 with stronger induction protocol failed to identify any difference in clinical score between the strains. The degree of difference in neuronal survival seen in VRA and TBI, if present to the same extent in EAE, can be difficult to detect with the rather insensitive clinical score scale used in EAE. Neuronal counts are much more complicated in EAE, since sites of lesions will differ between rats, and a certain limited area cannot be validated by its own. It is possible that difference in neuronal susceptibility is too small to be picked up with the clinical score in EAE that is affected by many more factors than only neurodegeneration. In addition, the sources of variation are larger in EAE compared to the nerve injury models.

To evaluate disease severity by other means than clinical score, measurable markers as antibody titres were used. Possibly increased tissue damage could lead to more autoantigens being released and a stronger immune response. Indeed, we found a significant difference in antibody titres towards neurofascin in CSF between the strains. Neurofascin is a protein that exists in two isoforms, located in myelin and myelinated axons respectively. Injection of neurofascin antibodies in adoptive transfer has previously been shown to exacerbate EAE and further elevated antibody titres have been shown in approximately 20% of MS patients, particularly in progressive disease [198]. A similar pattern as seen in MS, with a subgroup showing highly elevated CSF titre, is seen in the MOG-EAE experiment in paper IV. Notably, all rats with highly elevated levels all belong to the DA group that has more neuronal loss in VRA and TBI. There is also somewhat higher total IgG-titres in CSF and anti-MOG titres in serum of DA rats.
Figure 8: (Right) Anti-neurofascin antibodies in MS show high levels in a subpopulation of individuals and higher titres is especially seen in progressive disease (MS-CP)[199]. Reprinted with permission from *J Exp Med*. (Left) Results from anti-neurofascin titres in rat CSF after EAE show a similar pattern with a part of the individuals displaying high titres. Notably, all rats with highly elevated titres belong to the DA group.

This led to the design of an association study between various MS parameters and GSTA4 genotype in a large case/control material. Genotype was tested for association to case/control as well as MSSS, a severity score, and intrathecal antibody production. As expected from the size of the material, there was no strong association. However, suggestive associations to one SNP for IgG-index/presence of OCB and another for MSSS-score were detected. A haplotype showed a somewhat stronger association for MSSS score. Unfortunately, no pure measurement of neurodegeneration, such as for example a reliable biomarker, could be assessed since that type of information is not available. The ultimate data would be MRI estimates of brain grey matter atrophy, which likely would best represent degree of neuronal loss. However, no such material is available at present time.

A small Elisa experiment on CSF from MS patients and ONDs (data not included in the manuscript) showed no difference in average levels of GSTA4, but a large degree of variation within both groups. Since the hypothesis of variable sensitivity to HNE due to GSTA4 levels concerns levels within CNS neurons, expression analysis in human samples versus healthy controls is not possible. CSF might be a good way to depict CNS protein levels, and the preliminary data indicate a large variation. Inter-individual variation in human samples have also been found in hepatocytes [196]. However, interpretation of GSTA4 levels in CSF is unclear, since there is no data on extracellular GSTA4 in the CNS, although there are indications of a role of extracellular GSTA in the lung [200].
A role of *Gsta4* variation for disease severity in both MS and EAE must at this stage be considered as suggestive. In EAE, I believe that during the relatively short duration with an acute attack an effect of Gsta4 is likely have a low penetrance. In VRA and TBI, a clear difference in neuronal counts is seen with stereology based counts. However, this represents an experimental approach with far lower inter-individual variation within groups than EAE and also a much more precise measurement of neuronal cell death. To rule out a functional role of *Gsta4* in EAE, I believe that a stronger down regulation, likely a knock-out rat, is necessary.

In association to MS severity, a measurement that more purely depicts neurodegeneration, like grey matter atrophy, probably has a better chance to correlate to *GSTA4* variation. Although, MSSS fairly well depicts disease severity and progression in historical cohorts, there are other factors that influence the degree of neurological handicap, in particular the introduction of disease modifying treatments. However, the association data encourage further studies on GST activity in MS with relation to severity and progression. The HLA-locus, known to be the main risk gene for disease, is also in some studies seen to influence severity [201, 202]. Perhaps stratification for HLA and possibly other disease loci is needed in larger materials to increase the penetrance for *GSTA4* effect.

6.4 THE FORWARD GENETICS APPROACH

This study has utilized an unbiased genetic approach, often termed forward genetics, to identify a mechanism with biological relevance for neuronal death. The methodology has the advantage that it pinpoints mechanisms and can create new hypothesis not based on previous knowledge. This is in contrast to the hypothesis driven research, where a study is designed based on an assumption from previous findings and data. The forward genetics approach also has the advantage to study natural genetic variation, likely more relevant for human disease than for example a knock out approach.

However, it is time consuming and also a work intense way to find a mechanism of relevance that does not necessarily need to be novel data. From the first publication of the VRA1 QTL until publication of *Gsta4* as the likely underlying gene in paper II of this thesis, 8 years passed. This finally points towards regulation an established pathway for neurodegeneration. HNE toxicity has previously been extensively studied in neurodegenerative disorders and *in vitro* experiments and found to induce cell death in a concentration dependent manner. Further, biochemical studies have identified Gsta4-4 as extremely efficient enzyme in reducing HNE, and from knock out studies it is also regarded as the main enzyme catalysing the reduction in the CNS.
However, the main finding which I have also tried to emphasize is that there is a natural genetic variation in this pathway that to a large extent affect neuronal survival. This data now encourage further studies on this variation both in experimental models of other neurodegenerative disorders as well in human disease.

6.5 IS GSTA4 THE RIGHT GENE?

The smallest congenic described still has 35 protein coding genes. Each of these genes, or a combination of genes, could possibly explain the increased nerve cell survival seen in this strain. Although extensive recombinant breeding has been conducted, a congenic with only one or a few genes has not been achieved. There is of course a risk that another gene could alone or together with Gsta4 underlie the observed effect in the congenic rats. Since single gene congenics are extremely rare, identifying underlying genes mostly has to be based on a series of indications that together makes it unlikely that another gene is causative.

The main finding that led to the conclusion that Gsta4 is the right gene is the eQTL mapping results. This showed that only one cis-regulated gene exist in the congenic fragment. This together with the involvement in an established pathway for neurodegeneration, the cellular localization within neurons and a direct effect of the congenic on HNE-injection makes it unlikely for Gsta4 not to be of relevance for the trait. However, for example genetic variation that affects molecular functions and not expression levels in other genes could possibly have an effect that we have not detected.

It is important to point out that formal proof requires exact definition of the genetic variability underlying VRA1 and ideally the development of a single gene congenic. Even if a knock out generally do not contribute to the finding of effect of natural variation, such a strain could be used here as a prof of a relation between Gsta4 and nerve cell survival in our models.
7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In summary, this thesis highlights that naturally occurring genetic variation in a population to a large extent affect the degree of neuronal cell death in the CNS. It further points out a certain mechanism and gene that encourage future studies. Below, I try to summarize the most essential findings together with my opinion on important future steps to further validate the relevance of this genetic variation.

- Genetic mapping

Degeneration of neurons in the CNS after injury is highly dependent on genetic factors and here a relationship between capacity to detoxify reactive breakdown products of lipid peroxidation and degree of neurodegeneration is highlighted. Gsta4 is highly expressed in neurons and regulated between different inbred rat strains and higher levels of Gsta4 is associated with better neuronal survival in experimental models. The effect is seen in three different crosses and reproduced in congenic strains, where the smallest positive fragment contains only 35 genes.

Further reduction of the congenic fragment in order to get a single gene congenic only containing Gsta4 and no or few other genes in the fragment would be the ultimate proof that Gsta4 is the right gene underlying the trait. Extensive recombinant breeding has been done over the last years and there is now a recombinant from the R5 that further reduces the congenic interval by approximately 10 genes that can be used for further studies. Further reduction will be a very extensive work, both in terms of number of animals, genotyping and time, yet with unknown certainty of success. An alternative approach would be to make a genetically modified knockout rat; a technique that has been used in mice for decades but just recently has become available in this species [203, 204]. An approach that is considered is to create a partial knock out, where the levels of expression of Gsta4 could be regulated. This could prove an effect of variable expression level and also enable stronger down regulation than seen in DA versus R5. This is likely to be beneficial for the study of a Gsta4 effect in EAE where the phenotype is less well defined and the degree of inter-individual experimental variation is larger than in the neurodegenerative models.

The second region previously detected to affect neurodegeneration, VRA2, is still a suggestive region. The eQTL mapping revealed interesting candidate genes also here, but data is still too speculative for publication. However, just adjacent to the G10 intercross linkage peak, there is a cis-regulation of the gene Trp53inp1, an anti-proliferative and pro-apoptotic gene [205] involved in p53 dependent stress response, that has been implicated in diabetes [206] and as a tumour suppressor [207]. It has
further been shown to be involved in the antioxidant role of p53 [208], and mice deficient in TRP53inp1 are shown to have an increased oxidative load [209]. However, future studies are needed to evaluate the role of VRA2 and the underlying genetic variance, ideally with another model with better penetrance for the effect.

- **The mechanism in rat studies**

  The replication of an effect on neuronal survival originally observed in VRA to TBI indicates that this likely is a mechanism of general relevance for severity in various acute nerve injuries. Direct injection of HNE into the cortex is associated with lower degree of neuronal damage in rats displaying higher Gsta4 levels in the neurons. Further, a model of neuroinflammation, EAE, indicates that Gsta4 also might play a role for development of antibody response towards CNS autoantigens.

  The congenic strain described in this thesis could be used also in other models. It is planned to be tested in a model of Parkinson’s disease, which can provide further support for a general relevance of variations in this detoxification mechanism. Further, the fact that Gsta4 is highly expressed and located within neurons in the CNS will be of importance for interpretation of expression data from other studies and models. While most previous findings have dealt with the degree of oxidative burden and regulation of production of free radicals, these findings show variation in neurons ability to resist the same amount of oxidative burden.

  An important step in further studies will be to define a quantitative measure of HNE-protein complexes, and neuronal survival, ideally under controlled equal oxidative insult. Likely, in vitro studies of neuronal cultures would be a favourable approach were neurons from DA and R5 can be directly exposed HNE and other oxidants. These would possibly also enable quantitative measurements of HNE-modified neuronal proteins in high concentration. So far we have not successfully been able to quantify this given the low levels in our in vivo models.

  The mechanism of increased immunogenicity towards HNE modified proteins will also further be studied. Previous studies have shown that MDA modified MOG is more immunogenic and results in a more severe EAE disease course [124], and it not too speculative to believe that HNE-modification could have the same properties. Since HNE naturally modifies a wide range of proteins, an interesting approach would be induction of EAE with whole spinal cord, with and without in vitro modification of the homogenate. This would depict the wide spectrum of HNE-modified antigens that is likely to exist in human disease.
• **Relevance for human disease**

An association study to severity and intrathecal antibody response in MS show that genetic variation in *GSTA4* possibly can have an effect for disease severity. We have also in a small pilot test seen that there is a large variation in GSTA4 levels in CSF between individuals. These data encourage further investigation of a role for genetic variation of *GSTA4* in neurodegenerative and autoimmune disorders. Several lines of evidence suggest that generation of reactive oxygen species is of importance in this context. In light of this, the notion that higher levels of Gsta4 in CNS neurons correlate with better survival is of high interest, since this suggests yet another level where this important pathway may be regulated, indicating coping capability for oxidative stress and lipid peroxidation by-products as a key mechanism.

In terms of the combined effect of genetic risk factors for disease; my standpoint is that it would be of great value for understanding of disease mechanisms to evaluate combined effects of genetic variation in lipid peroxidation pathway. For example variation in *SOD1* which leads to increased oxidative stress in combination with certain *Gsta4* variants giving a low detoxifying capacity. This is an approach that should be of interest in large experimental populations where genetic manipulation of multiple genes is possible as well as for interaction analysis in human disease association studies. In theory, these genetic factors together would be a setting where combined effects could result in multiplicative disease risk as is expected for complex neurodegenerative disorders.
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