From γ-secretase in APO-SUS/-UNSUS rats to translational research in complex human disorders

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General introduction

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In societies from all over the world, we find human beings afflicted with a mental illness. The group of mental disorders can be divided into two main subgroups: neurodegenerative diseases and neurodevelopmental disorders. Neurodegeneration is the state in which cells of the brain and spinal cord are damaged or lost. Alzheimer's disease, Huntington's disease and Creutzfeldt-Jakob disease (mad cow disease) are examples of neurodegenerative diseases. In this thesis, the focus will be on the other subgroup, the neurodevelopmental disorders. The study will deal with a rat model for neurodevelopmental disorders (part A) and translational research in human (part B). This introductory chapter summarizes the current knowledge of the aetiology of complex neurodevelopmental disorders and the strategies followed to unravel its causes. Besides the genetic component that is clearly involved, also the environmental and epigenetic factors contributing to the pathology of neurodevelopmental disorders are briefly addressed.

Neurodevelopmental disorders and genetic aetiology

The relatively new term neurodevelopmental disorder includes a group of disorders with severely affected behavioral features caused by alterations in early brain development. Most neurodevelopmental disorders are associated with a life-long endurance and have a severe impact on normal brain functioning, leading to affected behavior often resulting in large economical, emotional and physical problems, not only for the individual but also for the family and society as a whole. The various neurodevelopmental disorders show similar features, including brain dysfunctioning (such as difficulties in sensor and motor systems, problems with speech and language) and a number of cognitive impairments (e.g. in learning and organizational skills). Schizophrenia, autistic disorders, attention deficit hyperactivity disorder (ADHD), bipolar disorder, mental retardation and Tourette's syndrome are some of the more common neurodevelopmental disorders, but also Rett syndrome, immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome and X-linked alpha thalassemia/mental retardation (ATR-X) syndrome are considered neurodevelopmental disorders (table 1).

Neurodevelopmental disorders can be divided into four subgroups, based on their (mostly hypothetical) genetic aetiology (table 1). The first subgroup is characterized by aneuploidy (an abnormal number of chromosomes). The most well-known neurodevelopmental aneuploidy is Down's syndrome with a trisomy of chromosome 21. Disorders of the second subgroup contain chromosomal micro-deletions, such as the deletion of chromosomal region 7q11.2 (which harbours more than 20 genes) in William's-Beuren syndrome. In each neurodevelopmental disorder of the third subgroup, only a single gene is affected. For example, the fragile X syndrome is a genetic disorder caused by a mutation (CGG repeat expansion) of the fragile X mental

Table 1. Neurodevelopmental disorders and their genetic aetiologies

Group	Disorder	Genetic aetiology
I (Aneuploidy)		
	Down's syndrome	Trisomy of chromosome 21 (OMIM #190685).
II (Micro-deletion)		
	Prader-Willi syndrome / Angelman syndrome	~4 Mb deletion (~7 genes) of chromosome 15q11-q13 (OMIM #176270 and #105830).
	Smith-Magenis syndrome	Deletion (3.7 Mb) of chromosome 17p11.2 (OMIM #182290).
	DiGeorge/velo-cardio-facial syndrome	Hemizygous deletion (1.5 to 3.0-Mb) of chromosome 22q11.2 (OMIM #188400 and #192430).
	William's-Beuren syndrome	Deletion of chromosomal region 7q11.2 (OMIM #194050).
III (Single-gene defect)		
	ATR-X syndrome	Mutations in the ATR-X gene on the X-chromosome (OMIM #301040)
	Barth syndrome (X-linked cardioskeletal myopathy and neutropenia)	Mitochondrial functional impairments due to the tafazzin (TAZ) gene on chromosome Xq28 (OMIM #302060).
	Fragile-X syndrome	CCG repeat expansion of the FMR1 gene (OMIM #300624).
	ICF syndrome	Mutations in the DNA methyltransferase 3B (DNMT3B) gene on chromosome 20 (OMIM #242860).
	Neurofibromatosis	Mutations or deletion (~1.5 Mb) in the neurofibromin gene on chromosome 17q11.2 (OMIM +162200).
	Rett syndrome	Mutations in the MeCP2 gene on the X-chromosome (OMIM #312750).
	Smith-Lemli-Opitz syndrome	Mutations in the gene encoding sterol delta-7-reductase (DHCR7) on chromosome 11q12-q13 (OMIM #270400).
IV (Multifactorial)		
	Addictive disorders	
	Attention deficit (hyperactivity) disorders	
	Anxiety disorders	
	Asperger's disorder	
	Autistic disorders	
	Bipolar disorder	
	Depressive illness	
	Dyslexia	— Multiple genes (?)
	Eating disorders	
	Epilepsy (seizure disorder)	
	Fetal alcohol syndrome	
	Hydrocephalus	
	Mental retardation	
	Schizophrenia	
	Spina bifida	
	Tourette's syndrome	

OMIM: Online Mendelian Inheritance in Man

retardation 1 (FMR1) gene on the X chromosome. The neurodevelopmental disorders with a complex aetiology, such as autism and schizophrenia, comprise the fourth subgroup and are thought to be caused by (a combination of) genetic, environmental and epigenetic factors. This chapter focuses on the neurodevelopmental disorders with a complex aetiology and the current thoughts on their genetic, environmental and epigenetic aetiologies.

Identification of susceptibility loci and genes

Twin, family and adoption studies have revealed an unambiguous role for genetic factors in the aetiology of complex neurodevelopmental disorders that can even exceed an estimated heritability of 90% (in autism; table 2). Although a genetic component is thus clearly involved in the aetiology of a complex neurodevelopmental disorder, it is still elusive which gene (or genes) is responsible for its pathogenesis. Historically, the dopamine and also the glutamate neurotransmission system have often been implicated to play a role in neurodevelopmental pathogenesis. However, since many recently identified susceptibility genes have been found not to be related to either of the two neurotransmitter systems, restriction to these systems is no longer justified. To identify susceptibility genes and to better understand the pathophysiology of complex neurodevelopmental disorders, many studies utilizing genetic, biochemical, pharmacological, neurological and cognitive neuroscience techniques have been performed. In this section, the genetic approaches that have been used to identify risk factors at specific loci and genes are summarized.

Linkage studies

Linkage analysis is a method to locate disease-related loci using DNA markers across the genome that travel with a disease within families. The main advantage of linkage analysis is that it involves family-based analysis, and thus eliminates the problem of ethnical stratification. However, linkage analysis has a relatively low power to detect small-effect variations (Risch and Merikangas, 1996).

Association studies

Numerous association studies have been performed to test for association between genetic variations and neurodevelopmental disorders (Craddock and Owen, 1996; Owen and Craddock, 1996). Compared to linkage analysis, one important advantage of association studies concerns its improved power when equal cohort sizes are used (Risch, 2000). In association studies, the genotype or allele frequencies of genetic variations between patients and controls (non-related individuals; case-control design) or between parents and their offspring (related individuals; family-based design) is compared. For the case-control design, a more than by chance predicted

difference in the frequency of a single-nucleotide polymorphism (SNP) between the cases and controls indicates that the specific polymorphism may increase or decrease risk for the disorder, or is in linkage disequilibrium with a nearby genetic variant. The frequencies of genetic varations may vary among individuals from a different geographical or ethnical background and therefore a well-defined cohort is necessary. For family-based association studies, the parents function as the controls for the affected offspring (so-called trio-study). If the SNP is transmitted from the parents to the offspring as expected by chance alone, no association with the disorder is present. Transmission of the SNP at a higher degree than expected by chance suggests association of the genetic marker with the affected phenotype.
Having decided on the study design and study samples, the next step is to select appropriate candidate genes. In general, a gene is selected with some a priori relationship with the disorder, based on its localization (i.e. the gene is located in a chromosomal region with a significant linkage), or proposed function in the pathogenesis of the disorder (e.g. the gene belongs to the dopamine or serotonin pathway in association studies for schizophrenia pathogenesis). Genome-wide association (GWA) studies can now also be performed, whereby large numbers of DNA polymorphisms are analyzed in one experiment.

Genomic copy number variations
Recently, it became clear that besides mutations and SNPs (both coding and non-coding alterations) also genomic rearrangements and gene-dosage imbalances (duplications, deletions and inversions) play a role in the pathogenesis of a number of nervous system disorders (reviewed by Lee and Lupski, 2006) (figure 1). These structural variants are common and ubiquitous in the genome and can range from kilobases to megabases in size. The human genome contains at least 1447 copy-number variants (CNVs), covering 360 megabases and comprising 12% of the genome (Redon et al., 2006). Previous knowledge of CNVs in relation to diseases was limited due to insufficient methods to detect CNVs. Only large CNVs detected with cytogenetic techniques, such as G-banding (Giemsa staining) and fluorescence in situ hybridization, have been previously identified. The advent of high-resolution genome-wide methods has significantly improved the power to detect CNVs.
At present, one of the most attractive techniques to detect CNVs is comparative genome hybridization (CGH) using DNA microarrays containing genomic DNA probes (e.g. bacterial artificial chromosome clones, cDNA clones or oligonucleotides). The CGH technology allows a genome-wide screening with a relatively high resolution (with the resolution depending on the number, distribution and lengths of the probes present on the array), and may be particularly useful for the identification of CNVs that are too small to detect via routine cytogenetic analyses. Another type of array

that can be used for detecting CNVs is the genome-wide SNP array. Besides normal SNP analysis (i.e. the identification of a single-base polymorphism), these arrays also give intensity information and thereby the corresponding copy number of a genomic region. Once CNVs have been detected, studies for locus-specific CNV association have to be designed, such as targeted quantitative and semiquantitative PCR, multiplex ligation-dependent probe amplification or dynamic allele-specific hybridization.

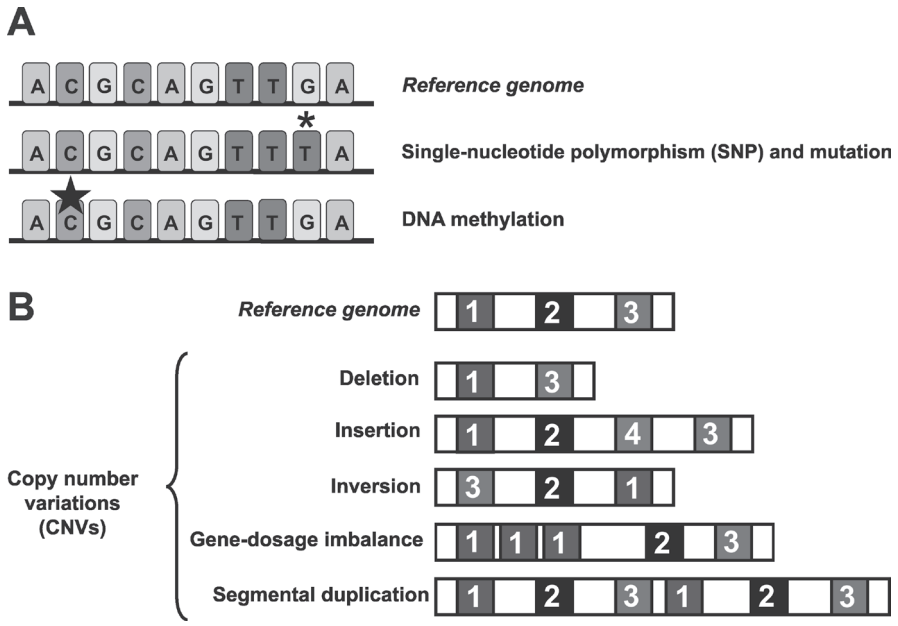

Figure 1. *Genetic and epigenetic variations in the genome. (A) A single-nucleotide polymorphism (SNP) or mutation (indicated by an asterix) represents a variation of a single base pair in the genome. SNPs are thought to be mutations that have been successful enough to remain within a population with a significant prevalence (>1%). The epigenetic variation DNA methylation (indicated by a star) represents addition of a methyl group to the cytosine within the sequence CG. (B) Copy number variations (CNVs) include genomic rearrangements and gene-dosage imbalances, and can range from kilobases to megabases in size (figure B based on Check, 2005).*

mRNA and protein expression profiling

The principle of mRNA and protein profiling is to identify genes that are differentially expressed in selected tissues of cases and (matched) controls. One of the promising strategies is the use of human post-mortem brain tissues, from which nowadays good quality mRNA can be extracted for microarray analysis (Tkachev et al., 2003). However, when performing such studies one has to be aware of the possibility that

the differential expression could be caused by years of medicine usage or by pre- or post-mortem artifacts.

Animal models

The use of animal models for the analysis of complex neurodevelopmental disorders appears to be an attractive alternative to circumvent the problems encountered when human material is used for mRNA or protein profiling. However, animals do not exhibit higher-order functions, some of which may be associated with complex human disorders. Nevertheless, one can take advantage of specific characteristics (endophenotypes) of an animal model to study the genetic and environmental factors that lead to a particular phenotypic outcome.

Several categories of animal models may be employed, including models based on a behavioral selection (e.g. the endophenotype prepulse inhibition), on a pharmacological selection (e.g. the psychotic effects of drugs, such as amphetamine) or on brain lesions (e.g. animal models with disconnections of the hippocampus). In addition, genetic animal models – with targeted genetic manipulations of specific genes - can be used, including knockout, knockin and transgenic models. Genetically modified animals can be subjected to a whole battery of behavioral tests to understand the role of a gene in neurodevelopmental aetiology. Furthermore, such models can be used to study environmental manipulations, including maternal or chronic stress paradigms. An example of an animal model for neurodevelopmental disorders represent the so-called APO-SUS/-UNSUS rats, a model based on a pharmacological selection (Cools et al., 1990). A more detailed description of this model will be given at the end of this chapter.

Susceptibility loci and genes of complex neurodevelopmental disorders

In the past decades, an impressive amount of linkage and association studies have been performed. However, conclusive evidence from the numerous genetic linkage and association studies has not yet been obtained. The studies have continuously led to inconsistent and controversial findings. In the next paragraphs, the genetic aetiology of the complex neurodevelopmental disorders is summarized. However, because positive associations are often published more easily one has to realize publication bias and the fact that even results obtained by meta-analysis may represent false positives.

Autism

Autism has a prevalence of ~0.6% in the general population and is four times more prevalent in boys than in girls. Together with four other disorders (Asperger's disorder, childhood disintegrative disorder, Rett syndrome and Personality Disorder

Not Otherwise Specified) it belongs to the group of Pervasive Developmental Disorders (PDD). Autism is the most common PDD and usually appears during the first three years of life. Its symptoms include impairments in verbal and nonverbal communication, lack of social interaction, and restricted and stereotypical behaviour (Montes and Halterman, 2006). Though autism is one of the most hereditary disorders in psychiatry, with an estimated heritability of up to 90% (table 2) (Freitag, 2007), the search for susceptibility genes has proven to be complex. Until now, a number of chromosomal loci have been identified that may represent regions predisposing to autism, including regions on chromosome 1p12-p21.1, 1q21-q44, 2q24.1-q33.1, 3q21.3-q29, 4q21.3-q35.1, 5p12-p15.33, 6q14.3-q23.2, 7q21.2-q36.2, 10p12.1-p14, 10q23.3-q26.3, 13q12.13-q33.1, 15q13.1-q26.1, 16p12.1-p13.3, 17q11.1-q21.2, 19p13.11-p13.3 and 19q12-q13.12 (reviewed by Yang and Gill, 2007). Although most susceptibility regions have been studied in more detail via the candidate-gene approach (e.g. the Reelin gene on chromosome 7q22 and the serotonin transporter gene (SLC6A4) on chromosome 17q11.1-q12), no gene has been found to clearly contribute to autism susceptibility. Recently, the first GWA studies for autism have been reported with significant associations, including CNVs, found in several genetic loci (Lauritsen et al., 2006; Sebat et al., 2007; Szatmari et al., 2007), but the results have been inconclusive. Thus, despite the high heritability estimates for autism, its genetic aetiology still needs to be elucidated.

Table 2. Estimated heritability of complex neurodevelopmental disorders

Disorder	Estimated heritability	References
Autism	>90%	(Freitag, 2007)
Schizophrenia	80%	(Cardno et al., 1999)
ADHD	70%	(Faraone and Khan, 2006)
Epilepsy	70%	(Kjeldsen et al., 2002)
Drug addiction	70%	(Sullivan and Kendler, 1999)
Spina bifida	70%	(Jorde et al., 1983)
Bipolar disorder	63%	(Smoller and Finn, 2003)
Eating disorders	48-74%	(Bulik et al., 1998; Kortegaard et al., 2001)
Dyslexia	50-70%	(Hawke et al., 2006)
Alcohol addiction	50-60%	(Hiroi and Agatsuma, 2005)
Panic disorder	30-46%	(Fyer et al., 2006)
Posttraumatic stress disorder	30%	(True et al., 1993)
Obsessive-compulsive disorder	26-47%	(Clifford et al., 1984; Jonnal et al., 2000)
Anxiety disorders	30-40%	(Hettema et al., 2001)
Depressive illness	37%	(Kendler and Karkowski-Shuman, 1997)

Schizophrenia

Schizophrenia is a common mental disorder affecting approximately 1% of the population (Jablensky et al., 1987). It generally emerges between 16 and 30 years of age and is characterized by three main symptoms: cognitive disturbances, psychosis and negative symptoms (Kay and Opler, 1987). Unfortunately, there are no genetic markers available for diagnosing schizophrenia. Therefore, diagnosis can only be based on clinical symptoms using the Diagnostic and Statistical manual for mental disorders version IV (DSM-IV, 2000) or the International Classification of Disease version 10 (ICD-10, 1992).

The first genetic studies on schizophrenia date back from 1916 and addressed the question whether the disorder has a genetic aetiology. Many subsequent family, twin and adoption studies clearly revealed the importance of a genetic component in schizophrenia (Gottesman, 1991) with an estimated heritability of around 80% (table 2) (Cardno et al., 1999), but the responsible gene (or genes) is still elusive. Although many susceptibility loci have been identified, numerous inconsistent and controversial findings have been reported. The genes most often reported to be related to schizophrenia are the genes encoding disrupted in schizophrenia 1 (DISC1; 1q42.1), neuregulin-1 (NRG1; 8p12), dysbindin (DTNBP1; 6p22.3), D-amino acid oxidase activator (DAOA or G72; 13q34), D-amino-acid oxidase (DAO; 12q24), regulator of G-protein signaling 4 (RGS4; 1q23.3) and the dopamine-catabolizing enzyme catechol-O-methyl transferase (COMT; 22q11.21) (reviewed by Owen et al., 2005; Ross et al., 2006). However, relative risk effects of the variations range between 1.5 to 2.0, indicating only small-effect sizes. Recently, the first GWA study for schizophrenia using the Affymetrix GeneChip 500K Mapping Array Set on 178 schizophrenic patients and 144 controls has been reported. One SNP (rs4129148) close to the colony stimulating factor 2 receptor alpha chain gene (CSF2RA) on chromosome Xp22.32 and Yp11.3 showed association beyond the genome-wide significance threshold (Lencz et al., 2007). Independent replications to confirm this finding are however necessary.

Bipolar disorder

Bipolar disorder, also known as manic-depressive illness, is a severe mental disorder characterized by recurrent manic and depressive episodes causing dramatic mood swings. The prevalence of bipolar disorder is estimated to be 0.8-2.6% (Kato, 2007). Although some have their first symptoms in childhood, most patients develop episodes in late adolescence or early adulthood. Bipolar disorder patients show many clinical features that are similar to those of schizophrenic patients, such as age of onset, psychotic symptoms, episodic courses of illness and a lifelong endurance. However, also clear distinctions exist between the two disorders. For example, bipolar disorder

manifests as an impairment of mood, whereas schizophrenia is a primary disorder of cognition. Furthermore, most bipolar patients benefit from lithium therapy, whereas schizophrenics seldom do.

Twin and family studies have shown that bipolar disorder tends to run in families with an estimated genetic hereditary of 63% (Smoller and Finn, 2003). Interestingly, besides the shared clinical symptoms, bipolar disorder and schizophrenia may also share a genetic background (Berrettini, 2003). A number of promising susceptibility genes for schizophrenia have been reported to associate with bipolar disorder as well, including G72, DAO, DISC1, NRG1, RGS4, COMT, neural cell adhesion molecule 1 (NCAM1; 11q23.1), brain-derived neurotrophic factor (BDNF; 11p13) glutamate receptor, metabotropic 3 and 4 (GRM3; 7q21.1-q21.2 and GRM4; 6p21.3), glutamate receptor, ionotropic, N-methyl D-aspartate 2B (GRIN2B; 12p12), megalencephalic leukoencephalopathy with subcortical cysts 1 (MLC1; 22q13.33), synaptogyrin 1 (SYNGR1; 22q13.1) and solute carrier family 12 (potassium/chloride transporters), member 6 (SLC12A6; 15q13-q15) (reviewed by Farmer et al., 2007; Kato, 2007). Recently, The Wellcome Trust Case Control Consortium reported a GWA study on bipolar disorder using the Affymetrix GeneChip 500K Mapping Array Set and found one chromosomal region with strong evidence of association (16p12) and 13 regions with moderate association (2p25, 2q12, 2q14, 2q37, 3p23, 3q27, 6p21, 8p12, 9q32, 14q22, 14q32, 16q12 and 20p13) (Consortium WTCC, 2007).

Major depression

Like bipolar disorder, depression is a major mood disorder. Although many clinical aspects are comparable between major depression and bipolar disorder, a number of characteristics are different between the two disorders: depression is much more heterogeneous, has a higher environmental contribution and has a higher prevalence with an overall lifetime risk of 16.2% in the United States (Kessler et al., 2003). Since the genetic contribution to major depression is only ~37% (Kendler and Karkowski-Shuman, 1997) and the illness is highly heterogeneous, unravelling its genetic pathogenesis is extremely difficult. To date, no clear genetic risk factors for major depression have been identified. Most studies have focused on well-known polymorphisms that have been hypothesized to associate with other psychiatric disorders. For example, the Val66Met variant in the BDNF gene, the short allele of the SLC6A4 gene, and the Val158Met variation in the COMT gene have been studied in depression cohorts (Caspi et al., 2003; Massat et al., 2005; Schumacher et al., 2005), but the results are contradictory (Frisch et al., 1999; Gillespie et al., 2005; Surtees et al., 2007).

ADHD

ADHD was first described in 1845 and affects up to 1 in 20 children (Comings,

2001; Faraone et al., 2003). The principal problem for children with ADHD is the impairment to control their behaviour, due to inattention, hyperactivity and impulsivity. According to the DSM-IV guidelines, these symptoms should appear early in a child's life, before age 7, and should continue for at least 6 months, otherwise the diagnosis ADHD is not justified. Other disorders often accompany ADHD, including learning disabilities, oppositional defiant disorder, conduct disorder, Tourette's syndrome and/or depressive illness.
Twin studies have indicated a relatively high genetic contribution reaching an average of 70% (Faraone and Khan, 2006). Thus far, many candidate gene studies on ADHD have focused on the dopamine and serotonin pathways. Meta-analyses of the available data have suggested several of the genes belonging to either pathway to be involved in ADHD pathogenesis, including the dopamine receptors D4 (DRD4; 11p15.5) and D5 (DRD5; 4p16.1), SLC6A4, the dopamine transporter (DAT or SLC6A3; 5p15.3), the 5-hydroxytryptamine (serotonin) receptor 1B (HTR1B; 6q13), dopamine beta-hydroxylase (DBH; 9q34) and synaptosomal-associated protein of 25kDa (SNAP25; 20p12-p11.2) (reviewed by Faraone and Khan, 2006). In addition, recently a large candidate gene analysis was performed involving 1,038 SNPs and spanning 51 candidate genes (belonging to the circadian rhythm genes and the dopamine, norepinephrine or serotonin pathways) that confirmed association with DRD4 and DAT1 (Brookes et al., 2006), the two most replicated associations.

Tourette's syndrome
Tourette's syndrome (also called Gilles de la Tourette syndrome) is a neuropsychiatric disorder that occurs with an estimated prevalence of 1% among school-age children (Robertson, 2003), is characterized by multiple chronic tics (involuntary movements and vocalizations) and is often accompanied by other behavioural disorders, including ADHD and obsessive-compulsive disorder (OCD) (Comings, 2001). Although family and twin studies have suggested a contribution of genetic factors in Tourette's syndrome, its precise contribution rate remains unclear (Pauls, 2003). Until now, most association studies have focussed on candidate genes belonging to the dopaminergic pathway, and showed several positive associations for the DAT, monoamine oxidase A (MAOA; Xp11.3) and the dopamine receptors D2 (DRD2; 11q23), D3 (DRD3; 3q13.3) and D4 (Comings et al., 1991; Comings et al., 1993; Comings et al., 1996b; Grice et al., 1996; Gade et al., 1998; Rowe et al., 1998; Tarnok et al., 2007; Yoon et al., 2007). However, since not all subsequent replication studies were positive (Gelernter et al., 1990; Brett et al., 1993; Gelernter et al., 1993; Brett et al., 1995; Barr et al., 1996, 1997) and other genes were found to associate as well (Abelson et al., 2005), the contribution of the dopaminergic pathway to Tourette's syndrome remains to be established.

Dyslexia (reading disability)
Dyslexia affects 5–10% of school-age children (Shaywitz et al., 1990) and is characterized by problems with word recognition and spelling. Linkage studies have revealed a number of chromosomal susceptibility loci for dyslexia (1p34-p36, 2p16-p15, 3p12-q12, 6p21, 6q13-q16, 11p15, 15q21, 18p11 and Xq27) (reviewed by Caylak, 2007). Within and near these loci several genes have been studied using association analyses, resulting in a few candidate genes for dyslexia: dyslexia susceptibility 1 candidate 1 (DYX1C1; 15q21) (Taipale et al., 2003), roundabout Drosophila homolog 1 (ROBO1; 3p12) (Hannula-Jouppi et al., 2005) and doublecortin domain-containing protein 2 (DCDC2; 6p22.1) (Meng et al., 2005), but again the results are not conclusive and therefore the genetic aetiology of dyslexia is currently still unclear.

Epilepsy (seizure disorder)
Epilepsy is a heterogeneous group of disorders with abnormal electrical brain activity. In adults, temporal lobe epilepsy (TLE) is the most common form of epilepsy with an age of onset in late childhood or adolescence. In childhood, the most common form of epilepsy is febrile seizures (FSs), with a prevalence of 2-5% in Western countries and an estimated heritability of 70% (Kjeldsen et al., 2002). Genetic linkage analyses have identified a number of loci for familial FS, including the loci on chromosome 19p13.3, 2q23-q24, 5q14-q15 and 18p11.2 containing the genes encoding casein kinase I gamma 2 isoform (CSNK1G2; 19p13.3), sodium channel, voltage-gated, type I, alpha subunit (SCN1A; 2q24.3), G protein-coupled receptor 98 (GPR98; 5q13) and inositol(myo)-1(or 4)-monophosphatase 2 (IMPA2; 18p11.2), respectively (Johnson et al., 1998; Peiffer et al., 1999; Nakayama et al., 2000; Nakayama et al., 2004). Although linkage of these loci has been replicated in some other familial cases, only for CSNK1G2 and IMPA2 association was found in subsequent association analysis (Nakayama et al., 2004; Yinan et al., 2004). In addition, a number of other candidate genes have been identified via association studies, including the genes encoding cholinergic receptor nicotinic alpha 4 (CHRNA4; 20q13.2-q13.3), gamma-aminobutyric acid A receptor gamma 2 (GABRG2; 5q31.1-q33.1) and -beta 3 (GABRB3; 15q11.2-q12), interleukin 1 beta (IL1B; 2q14) and interleukin 1 receptor antagonist (IL1RN; 2q14.2) (Steinlein et al., 1997; Feucht et al., 1999; Kanemoto et al., 2000; Baulac et al., 2001; Tsai et al., 2002). However, the latter associations could not be replicated in subsequent cohorts (Tilgen et al., 2002; Nakayama et al., 2003; Mulley et al., 2004; Haspolat et al., 2005; Hempelmann et al., 2007).

Mental retardation
Mental retardation (MR) occurs in approximately 2-3% of the population in developed

countries (Chelly and Mandel, 2001). For the diagnosis MR a number of criteria have to be fulfilled, including an IQ lower than 70 and behavioural disabilities that are already evident in childhood. The underlying causes of MR can be diverse, varying from inborn causes such as Down's syndrome, Fragile X syndrome and fetal alcohol syndrome (these three causes are responsible for 30% of the MR cases (Batshaw, 1993)), but also malnutrition and problems during pregnancy or birth can increase the risk for MR (Hagberg and Kyllerman, 1983).
Although it is evident that a genetic factor is involved in the aetiology of MR and the genetic cause of a number of subtypes has been identified (e.g. trisomy of chromosome 21 in Down's syndrome), the majority of cases have an unknown genetic aetiology. Since MR has a clearly X-linked inheritance pattern and is more often found in males than females, variations in the X-chromosome may increase the risk for MR. A number of X-linked genes have been identified as susceptibility genes for MR, including fragile X mental retardation 2 (FMR2; Xq28), oligophrenin 1 (OPHN1; Xq12), p21 (CDKN1A)-activated kinase 3 (PAK3; Xq22.3-23), GDP dissociation inhibitor 1 (GDI1; Xq28), Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (ARHGEF6; Xq26), ribosomal protein S6 kinase, 90kDa, polypeptide 3 (RPS6KA3; Xp22.2-p22.1), interleukin 1 receptor accessory protein-like 1 (IL1RAPL1; Xp22.1-p21.3), tetraspanin 7 (TSPAN7; Xp11.4), methyl CpG binding protein 2 (MECP2; Xq28), acyl-CoA synthetase long-chain family member 4 (ACSL4; Xq22.3-q23) and aristaless related homeobox (ARX; Xp21) (Gecz et al., 1996; Allen et al., 1998; Billuart et al., 1998; D'Adamo et al., 1998; Carrie et al., 1999; Merienne et al., 1999; Kutsche et al., 2000; Zemni et al., 2000; Couvert et al., 2001; Meloni et al., 2002; Stromme et al., 2002). However, many other genes are likely linked to MR.

Addictive disorders
Many twin studies have been performed on addictive disorders (both alcohol and drug abuse), which indicated heritability levels of 50-60% in alcohol consumption (Hiroi and Agatsuma, 2005) and up to 70% in severe smoking (Sullivan and Kendler, 1999). Since the dopaminergic pathway plays a central role in the reward system, the genes involved in this pathway are thought to be susceptibility genes for addictive disorders. Indeed, a number of studies have identified polymorphisms in this pathway that infer susceptibility to addiction: genetic variations in the DRD2, DRD3, COMT and DAT1 genes have been reported to associate with smoking, alcoholism, cocaine abuse and heroin addiction (Blum et al., 1990; Noble et al., 1993; Muramatsu and Higuchi, 1995; Duaux et al., 1998; Comings et al., 1999; Tiihonen et al., 1999; Horowitz et al., 2000; Xu et al., 2004; Guindalini et al., 2006; Timberlake et al., 2006). Nevertheless, despite the large number of studies reporting association, meta-

analyses have shown that the effects are only weak or not significant (Munafo et al., 2004; Munafo et al., 2007).

Fetal alcohol syndrome

During pregnancy, alcohol use by the mother may lead to fetal alcohol syndrome (FAS) that occurs at a rate of 0.5-2 individuals per 1000 live births. A number of family, twin and animal studies have suggested a genetic component in FAS pathogenesis, one of the main candidate genes being the alcohol dehydrogenase 1B (ADH1B) gene located on chromosome 4q21-q23. However, whereas some studies report a protective effect for a number of ADH1B subtypes, others were not successful in reproducing these results (reviewed by Green and Stoler, 2007). Besides ADH1B, other candidate genes have been suggested as risk factors for FAS pathogenesis, such as the cytochrome P450 2E1 gene (CYP2E1; 10q24.3-qter) (Boutelet-Bochan et al., 1997; Rasheed et al., 1997).

Anxiety disorders

Panic disorder, OCD, separation anxiety, overanxious disorder, agoraphobia and other phobias all belong to the group of anxiety disorders and are relatively common (lifetime prevalence of 25% (Kessler et al., 1994)). Twin studies have indicated that a genetic factor is involved in anxiety disorders, but the genetic contribution to the disorders is only modest (30-40%) (Hettema et al., 2001). Yet, many linkage and association studies have been performed to determine the chromosomal locations or genes involved in the pathogenesis of the various subtypes of anxiety disorders. Panic disorder showed significant linkage to chromosomal regions 9q31, 13q and 22q (Hamilton et al., 2003; Thorgeirsson et al., 2003), for OCD linkage was reported to chromosome 1q, 3q27-28, 6q, 7p, 9p24, 10p15, 14 and 15q (Hanna et al., 2002; Shugart et al., 2006; Hanna et al., 2007; Samuels et al., 2007), and for other anxiety disorders linkage was observed for chromosome 14p (simple phobia) (Gelernter et al., 2003), 16 (social phobia) (Gelernter et al., 2004), 1q, 4q, 7p, 12q and 13q (neuroticism) (Fullerton et al., 2003) and 8p21-23 (harm avoidance) (Cloninger et al., 1998). Recently, also genome-wide linkage analyses on individuals with a broad anxiety phenotype rather than based on the DSM-IV anxiety disorder diagnosis have been performed and significant linkage was observed for chromosome 14 (Middeldorp et al., 2007) and 4q31-q34 (Kaabi et al., 2006).

Besides linkage analysis, many case-control design studies on candidate genes for anxiety pathogenesis have been performed. For panic disorder, a positive association was found for the serotonin receptors HTR1A (5q11.2-q13) and HTR2A (13q14-q21) (Inada et al., 2003; Rothe et al., 2004), COMT (Domschke et al., 2004), the neuropeptide cholecystokinin (CCK; 3p22-p21.3) (Miyasaka et al., 2004), the

adenosine A2a receptor (ADORA2A; 22q11.23) (Deckert et al., 1998), MAOA (Deckert et al., 1999), the nuclear transcription factor cAMP-responsive element modulator (CREM; 10p11.21) (Domschke et al., 2003), the peripheral benzodiazepine receptor (PBR or TSPO; 22q13.31) (Nakamura et al., 2006), glutamic acid decarboxylase 1 (GAD1; 2q31) (Hettema et al., 2006), diazepam binding inhibitor (DBI; 2q12-q21) (Thoeringer et al., 2007), calmodulin-dependent protein kinase kinase b (CaMKKb; 12q24.2) (Erhardt et al., 2007) and angiotensin-converting enzyme (ACE; 17q23.3) (Olsson et al., 2004). In addition, an association analysis of 90 SNPs located in 21 candidate genes revealed eight SNPs to be associated with panic disorder (located in the CCK, serotonin and dopamine systems), but all with a minor individual effect (Maron et al., 2005).

Besides association with panic disorder, a number of susceptibility genes have been found to associate with other subtypes within anxiety disorders as well, such as the serotonin system in OCD and neuroticism (Lesch et al., 1996; Camarena et al., 2004; Lochner et al., 2004; Meira-Lima et al., 2004; Schinka et al., 2004; Sen et al., 2004), MAOA in generalized anxiety disorder and neuroticism (Eley et al., 2003; Tadic et al., 2003), COMT in neuroticism and phobic anxiety (Eley et al., 2003; McGrath et al., 2004) and BDNF in anxiety-related personality traits (Lang et al., 2005; Hunnerkopf et al., 2007).

Posttraumatic stress disorder

Posttraumatic stress disorder (PTSD) can occur in a subset of individuals exposed to extreme traumatic events (Nemeroff et al., 2006), and has a lifetime incidence of ~9–15% (Kessler et al., 1995; Breslau et al., 1998), and an estimated genetic inheritance of ~30% (True et al., 1993). Susceptibility genes for PTSD have not yet been identified, but to date the number of individuals screened is low, while the few genetic studies that have been performed mainly focussed on key candidate genes, including BDNF, neuropeptide Y (NPY; 7p15.1), the glucocorticoid receptor (NR3C1; 5q31.3), and components of the serotonin and dopamine pathways (Comings et al., 1996a; Lappalainen et al., 2002; Bachmann et al., 2005; Lee et al., 2005; Zhang et al., 2006a).

Eating disorders

Anorexia and bulimia nervosa are two major eating disorders with still unknown risk factors. For a long time, eating disorders have been considered to be caused by sociocultural factors. However, it has recently become clear that also genetics may play a substantial role in its aetiology. Family and twin studies have shown that heritability estimates for eating disorders vary from 48% to 74% in anorexia nervosa and from 55% to 83% in bulimia nervosa (Kendler et al., 1991; Bulik et al., 1998;

Klump et al., 2001; Kortegaard et al., 2001). Since serotonin plays an important role in mood and feeding, genetic variations in the serotonergic pathway are thought to lead to eating disturbances. Indeed, a number of positive associations with the serotonin receptors HTR2A and HTR2C (Xq24), and also with the serotonin transporter gene have been reported (Collier et al., 1997; Di Bella et al., 2000; Westberg et al., 2002), however, replication was not always successful (Campbell et al., 1998; Burnet et al., 1999). Furthermore, associations were found for BDNF (Koizumi et al., 2004; Ribases et al., 2004), the growth hormone secretagogue receptor (ghrelin receptor or GHSR; 3q26.31) (Miyasaka et al., 2006) and COMT (Frieling et al., 2006; Mikolajczyk et al., 2006).

Spina bifida

Spina bifida is caused by unsuccessful closure of the neural tube during early development (between embryonic day 17 and 30) and occurs with a frequency of 1-2 cases per 1000 births. The exact aetiology of spina bifida is poorly understood, but it is clear that both genetic and environmental factors are involved (Frey and Hauser, 2003). Since individuals with spina bifida often die prenatal or early postnatal and thus hardly any families exist with several affected members, this disease could well be the most difficult complex disorder to study at the genetic level. Based on animal and epidemiological studies, genes involved in folic acid (folate), vitamin B12 and homocysteine metabolism, or genes involved in neurulation have been hypothesized to play a role in spina bifida genesis (reviewed by Padmanabhan, 2006). However, until now, only a few genes have been reported to represent risk factors for spina bifida, including 5,10-methylenetetrahydrofolate reductase (MTHFR; 1p36.3) (Whitehead et al., 1995), methionine synthase reductase (MTRR; 5p15.3-p15.2) (van der Linden et al., 2006), platelet-derived growth factor receptor alpha (PDGFRA; 4q11-q13) (Joosten et al., 2001) endothelial nitric oxide synthase 3 (NOS3; 7q36) (Brown et al., 2004) protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1; 6q24-q25) (Zhu et al., 2006) and cofilin 1 (non-muscle) (CFL1; 11q13) (Zhu et al., 2007).

Hydrocephalus

Hydrocephalus occurs at a frequency of approximately 0.5 in 1000 births (Fernell et al., 1986; Halliday et al., 1986) and is characterized by abnormal flow or resorption of cerebrospinal fluid. It is considered a heterogeneous complex disorder (Willems, 1988) with genetic and environmental aetiologies (Stoll et al., 1992; Haverkamp et al., 1999). Although approximately 37% of the hydrocephalus cases have a possible genetic aetiology (Haverkamp et al., 1999), clear susceptibility genes for hydrocephalus have not been identified yet. Studies in animal models have suggested several loci as susceptibility regions for hydrocephalus, but these regions have not yet

been reported as susceptibility regions in human (reviewed by Zhang et al., 2006b).

Complex neurodevelopmental disorders and the environment

Since in general complex neurodevelopmental disorders have an estimated heritability lower than 100% (table 2), their aetiology includes another component that is thought to be primarily the environment (e.g. stressful life events). Numerous factors acting during early development of a foetus may contribute to the genesis of a neurodevelopmental disorder, including insufficient maternal nutrition, daily smoking, viral infection and repeated psychological stress (Schroeder, 2000). Most environmental vulnerability factors are however difficult to assign and quantify.

The type and timing of the early environmental risk factors to which an organism is exposed appear to determine the phenotypic outcome. For example, a prenatal exposure of 9-days-pregnant mice to a sublethal intranasal administration of influenza virus led to both short-term and long-lasting deleterious effects on the developing brain structures and to abnormal behavior in the offspring of the mice (Fatemi et al., 2002). Besides risk factors during early (prenatal) development, also obstetrical complications, including the use of resuscitation or an incubator, premature membrane rupture, diabetes, rhesus incompatibility, bleeding, preterm birth or caesarean birth, may increase the vulnerability to neurodevelopmental disorders (Curatolo et al., 1995; Boog, 2004).

One obvious gene-environment link concerns the season in which birth took place. An excess of winter-spring births in bipolar disorder and schizophrenia has been observed (Torrey et al., 1996). A similar tendency has been found in schizoaffective disorder (December-March), major depression (March-May) and autism (March) (Torrey et al., 1997). Besides the season of birth, also the place of birth is thought to be associated. Urban–born (and brought-up) subjects are more susceptible to neurodevelopmental disorders than rural-born (and brought-up) subjects (Torrey et al., 1997; Mortensen et al., 1999). Furthermore, risk factors like immigration and adoption may contribute to the development of psychiatric disorders (Cantor-Graae and Pedersen, 2007; Yearwood et al., 2007).

Gene-environment interactions in complex neurodevelopmental disorders

One of the reasons that the genetic and environmental factors in complex neurodevelopmental disorders are difficult to define is the fact that the two factors may interact. Moreover, such an interaction may be complex and act at various levels. For instance, genetic and environmental factors may have an additive effect, genetic factors may affect the influence of the environment on a phenotype or environmental factors may modulate the expression of genetic variants.

An example of a gene-environment interaction concerns the influence of stressful

life events on depressive individuals with a functional polymorphism in the promoter region of the serotonin transporter gene. Individuals with the short allele have been found to respond differently to stressful life events (e.g. childhood maltreatment) and as such are more vulnerable to develop depressive symptoms than individuals with the long allele (Caspi et al., 2003). A second example of gene-environment interaction is the valine/methionine polymorphism (SNP rs4680) in the COMT gene. Upon cannabis use, individuals carrying the valine allele have a higher chance to exhibit psychotic symptoms and to develop schizophreniform disorders when compared to individuals with two methionine alleles (Caspi et al., 2005).

Complex neurodevelopmental disorders and epigenetics

Epigenetics is defined as heritable changes in gene expression patterns that occur without changing the DNA sequence itself (Wolffe and Matzke, 1999), and includes DNA methylation and posttranslational modifications of histone proteins. DNA methylation, i.e. a covalent binding of a methyl group to the 5-position of the cytosine ring within the sequence 5'-CG-3' (CpG), can be tissue- and cell-type specific and is found in all vertebrates, and many invertebrates and plants. CpG clusters with a minimum of 200 base pairs, a CG percentage greater than 50% and an observed/expected CpG ratio greater than 0.6 are called CpG islands. These islands are often found in gene promoter regions and can protect single CpGs within a CpG island from DNA methylation.

An apparent link between the methylation status and gene transcription levels has led to the speculation that alterations in the methylation pattern (epimutations) might contribute to altered gene expression. Such epimutations are thought to occur upon exposure to environmental risk factors, including early developmental stress. Since early embryos seem to be particularly sensitive to epimutations (Reik et al., 1993; Rideout et al., 2001), this factor should be considered for the aetiology of neurodevelopmental disorders. For instance, epigenetic alterations are responsible for a number of neurodevelopmental disorders with single-gene defects, such as Rett Syndrome, ICF Syndrome, Fragile X Syndrome and ATR-X Syndrome (Amir et al., 1999; Xu et al., 1999; Gibbons et al., 2000; Jin and Warren, 2000). A role for DNA methylation has also been proposed in connection with complex neurodevelopmental disorders. For example, spina bifida can be caused by a lack of folate (reviewed by Pitkin, 2007), a compound needed for the generation of S-adenosylmethionine (SAM) that donates the methyl group in the DNA methylation process. Also, some patients with depressive illness and schizophrenia display lower serum folate levels (Herran et al., 1999). Animal models further provide evidence for a possible link between epigenetics and neurodevelopmental disorders. Following a diet with L-methionine, a precursor in the biosynthesis of SAM, the reeler mouse (a model for

schizophrenia) showed increased promoter methylation of the reelin gene, reduced reelin expression and a declined prepulse inhibition of startle. These effects could subsequently be reversed by valproic acid, a mood-stabilizing drug used for treatment of epilepsy, bipolar disorder and schizophrenia (Tremolizzo et al., 2002). In addition, the adult offspring of rat mothers that showed high licking and grooming (LG) and arched-back nursing (ABN) (two forms of maternal behaviour in the rat that serve as the basis for the individuals programming of the stress response) are less fearful, have a lower hypothalamic-pituitary-adrenal response to stress, and have a lower DNA methylation status in the promoter region of the glucocorticoid receptor gene when compared to the offspring of low-LG and -ABN mothers (Weaver et al., 2004). Thus, alterations in epigenetic profiles may contribute to the generation of complex neurodevelopmental disorders.

APO-SUS and APO–UNSUS rat model for complex neurodevelopmental disorders

The genetic, environmental and epigenetic contributions to the aetiology of neurodevelopmental disorders illustrate that unravelling the pathogenesis of these disorders is highly complex. Although insights into the degree of the genetic contribution to the aetiology of neurodevelopmental disorders have been obtained, the identities of the genes involved and thus diagnostic markers are mostly lacking. The use of an animal model with (aspects of) neurodevelopmental disorders represents an interesting strategy to identify new genes and pathways responsible for neurodevelopmental pathogenesis. An example of such an animal model is the APO-SUS/-UNSUS rat model already mentioned above, a model based on a pharmacological selection. Rats from an outbred Wistar population were selected on the basis of their response to the dopamine D2 receptor agonist apomorphine (Cools et al., 1990). Upon injection of this drug, a bimodal distribution of the stereotyped gnawing response can be found. Approximately 40% of the Wistar rats showed a weak gnawing response (<10 counts/45 min) and a similar percentage showed an intense gnawing response (>500 counts/45 min). Through a specific breeding program, two rat lines representing the two extremes have been created, named the APO-SUS rats (apomorphine susceptible rats with an intense gnawing response) and the APO-UNSUS rats (apomorphine unsusceptible rats with a weak gnawing response) (Cools et al., 1990; Ellenbroek et al., 2000) (figure 2). This breeding program has been carried out twice, with a ten-year interval, leading to the original APO-SUS and –UNSUS lines (with the breeding started in 1985) and the replicate APO-SUS and –UNSUS lines (breeding started in 1995). Extensive characterization of the APO-SUS and –UNSUS rat lines has revealed many differences which are not limited to the dopaminergic pathway, but also include behavioural, neurochemical,

immunological and endocrinological differences (table 3).
Interestingly, several phenotypical features of the APO-SUS rats strongly resemble characteristics of patients suffering from a neurodevelopmental disorder, suggesting that the APO-SUS/-UNSUS model may be a good model to study (aspects of) neurodevelopmental disorders. For instance, APO-SUS rats show abnormalities in information processing (Ellenbroek et al., 1995), a phenomenon also observed in patients suffering from schizophrenia, bipolar disorder, obsessive compulsive disorder, Tourette's syndrome, temporal lobe epilepsy with psychosis and posttraumatic stress disorder (PTSD) (Braff and Geyer, 1990; Swerdlow et al., 1993; Castellanos et al., 1996; Grillon et al., 1996; Pouretemad et al., 1998; Perry et al., 2001). In addition, APO-SUS rats have an altered hypothalamus-pituitary-adrenal (HPA) axis response to stress (Rots et al., 1995; Rots et al., 1996a), a characteristic also seen in patients with schizophrenia, mania, depressive illness, ADHD, anxiety disorders, PTSD and eating disorders (Carson et al., 1988; Kaye et al., 1988; Smith et al., 1989; Kaneko et al., 1993; Lammers et al., 1995; Schmider et al., 1995). Furthermore, it has been shown that both genetic and environmental factors can influence the APO-SUS/-

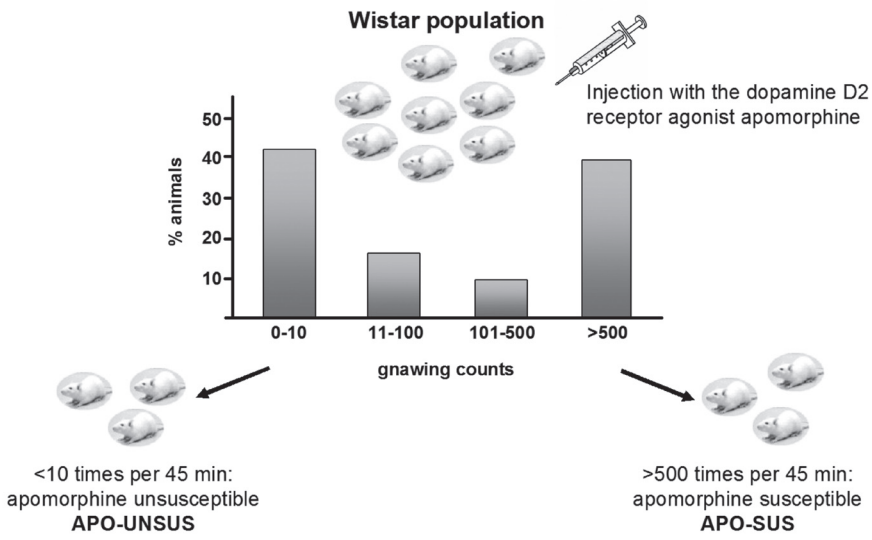

Figure 2. *Schematic representation of the generation of the APO-SUS and –UNSUS rat model. Rats from the Wistar population were injected with the dopamine D2 receptor agonist apomorphine and tested for their gnawing responses. Rats with a low gnawing score (<10 times per 45 minutes) and rats with a high gnawing score (>500 times per 45 minutes) were selected and used for breeding of the apomorphine-unsusceptible (APO-UNSUS) and apomorphine-susceptible (APO-SUS) rat lines, respectively (based on Cools et al., 1990).*

UNSUS phenotypes (Ellenbroek et al., 2000) as can also be seen in the pathogenesis of human neurodevelopmental disorders. Thus, the APO-SUS/-UNSUS rats may represent a valuable model in our attempt to unravel the genetic factors involved in the pathogenesis of disorders with a complex aetiology.

Outline of this thesis

This thesis focuses on candidate gene and pathway discovery in disorders with a complex aetiology, especially the neurodevelopmental disorders. Although numerous studies suggest that genetic variants play a significant role in the aetiology of complex disorders, in almost all cases the precise genetic background remains to be identified. A better understanding of the genes and pathways involved may be useful for the development of specific medicines and the application of disease-preventing strategies. The goal of the research described in this thesis is twofold and the thesis is therefore divided into two parts. Part A concerns a search for the molecular basis of the APO-SUS and -UNSUS rats. On the basis of the results obtained in the rat, we describe in part B human genetic association analyses in a number of complex disorders.

Part A. Rat model

Chapter A1 describes the search for gene transcripts which are differentially expressed in brain regions from the APO-SUS/-UNSUS rats. The expression profiles of hippocampal APO-SUS and –UNSUS rats have been analysed using microarray technology and revealed only one transcript, *Aph-1b*, to be differently expressed. Aph-1b is a component of the γ-secretase enzyme complex that is responsible for the proteolytic processing of a wide variety of type I transmembrane proteins and is involved in multiple (neuro)developmental pathways. The possibility of the γ-secretase signalling cascade as a susceptibility pathway for neurodevelopmental disorders is discussed.
Chapter A2 describes the expression levels of *Aph-1b* and its family members *Aph-1aS* and *-1aL* in APO-SUS and –UNSUS rats during development (starting from embryonic day 13). Besides the ontogenic expression levels for the *Aph-1* family, tissue- and time-specific cleavage of the γ-secretase substrate APP is presented.
Chapter A3 shows the expression levels of the *Aph-1* family in APO-SUS and –UNSUS rats in several tissues. In addition, cleavage of a number of γ-secretase substrates, including the APP superfamily, p75 neurotrophin receptor, ErbB4 and neuregulin-2, was found to occur in a tissue-specific manner.

Table 3. Phenotypic characteristics of APO-SUS rats compared to those of APO-UNSUS rats

Phenotypic characteristics	References
↑ apomorphine susceptibility	(Cools et al., 1990)
↓ noradrenaline immunoreactivity in nucleus accumbens	(Cools et al., 1990)
↑ hippocampal and pituitary mineralocorticoid receptor binding capacity	(Sutanto et al., 1992)
↑ metabolic activity in hippocampal area	(Cools et al., 1993)
↓ hippocampal dynorphin-B	(Cools et al., 1993)
↓ sensitivity for encephalitis and rheumatoid arthritis	(van de Langerijt et al., 1994)
↑ hypothalamic corticotropin releasing hormone mRNA	(Rots et al., 1995)
↑ basal plasma levels of ACTH	(Rots et al., 1995)
↓ basal plasma levels of free corticosterone	(Rots et al., 1995)
↑ ACTH and corticosterone plasma levels upon novelty	(Rots et al., 1995)
↓ prepulse inhibition of the acoustic startle response	(Ellenbroek et al., 1995)
↓ latent inhibition in a conditioned taste aversion paradigm	(Ellenbroek et al., 1995)
↑ hypothalamic synaptic density	(Mulders et al., 1995)
↑ TH mRNA in nigrostriatal and tuberoinfundibular system	(Rots et al., 1996b)
↑ dopaminergic D2 receptors in striatum	(Rots et al., 1996b)
↑ susceptibility to behavioural effects of dexamphetamine	(Cools et al., 1997)
↓ number of blood T cells	(Kavelaars et al., 1997)
↑ number of blood B cells	(Kavelaars et al., 1997)
↑ T_{H2} response upon infection	(Kavelaars et al., 1997)
↑ incidence of involuntary muscular contractions upon GABA activation	(Dirksen et al., 1997)
↓ hippocampal mossy fiber terminal fields	(Spooren et al., 1999)
↑ sensitivity to periodontitis	(Breivik et al., 2000)
↑ incidence of bursts of bilateral synchronous spike wave discharges	(de Bruin et al., 2000)
↓ alcohol intake under non-challenged conditions	(Sluyter et al., 2000)
↓ relaxation of mesenteric arteries upon b_2-agonist stimulation	(Smits et al., 2002)
↓ relaxation of mesenteric arteries upon a_2-agonist stimulation	(Smits et al., 2002)
↓ tumor growth	(Teunis et al., 2002)
↓ angiogenesis	(Teunis et al., 2002)
↓ contribution of nitric oxide to the vascular tone	(Riksen et al., 2003)
↓ recovery from gastric ulcerations	(Degen et al., 2003)
↓ number of natural killer cells in the spleen	(Teunis et al., 2004)
↑ fiber network and varicosities in nucleus accumbens	(van der Elst et al., 2005)
↓ speed of development	(Degen et al., 2005)
↑ alcohol consumption after an acute challenge	(van der Kam et al., 2005b)
↓ cocaine intake under habituated circumstances	(van der Kam et al., 2005a)
↑ cocaine intake under stressful circumstances	(van der Kam et al., 2005a)

Chapter A4 identifies additional differences between the APO-SUS and –UNSUS rats using arbitrarily primed-polymerase chain reaction. Besides the gene-dosage imbalance of the *Aph-1b* locus, additional genetic and epigenetic variations have been found to segregate with the APO-SUS/-UNSUS lines. We discuss the possibility that these newly identified variations may contribute to the complex phenotype and, as a consequence, suggest that *Aph-1b* might not be the only genetic factor responsible for the complex APO-SUS phenotype.
Chapter A5 describes CNVs other than the *Aph-1b* locus in the APO-SUS and –UNSUS rat genomes. By using CGH and (quantitative) genomic PCR analysis in APO-SUS and –UNSUS rats, we show eight new intragenic chromosomal regions to contain a CNV when comparing the genomes of the original and replicate APO-SUS and –UNSUS rats. A possible contribution of these CNVs to the complex APO-SUS phenotype is discussed.

Part B. Human

Chapter B1 describes association analysis of the γ-secretase pathway with premature atherosclerosis. A non-synonymous polymorphism in the human *APH1B* gene (Phe217Leu; rs1047552) was studied in a Caucasian case-control cohort for premature coronary atherosclerosis. Furthermore, the functional effect of this polymorphism *in vitro* is presented and the contribution of the γ-secretase signalling cascade in vascular pathogenesis is discussed.
Chapter B2 examines association of the functional Phe217Leu polymorphism in the human *APH1B* gene with the neurodevelopmental disorder epilepsy. We discuss the contribution of this polymorphism in epileptic seizures.
Chapter B3 describes association analysis of the *APH1B* Phe217Leu polymorphism in human immunodeficiency virus type 1 (HIV-1) infection. The results of two ethnical (Caucasian and South African) case-control cohorts are presented and implications for the contribution of the γ-secretase signalling cascade in susceptibility for HIV-1 infection is discussed.
Chapter B4 examines the influence of the *APH1B* Phe217Leu polymorphism on a number of disorders with a complex aetiology. Its role in susceptibility for schizophrenia, bipolar disorder, autism, ADHD, dyslexia, depression, rheumatoid arthritis, celiac disease and cancer (colorectal, throat, prostate and lung cancer) is presented and discussed.

In the general discussion, the results of the studies reported in this thesis are discussed and placed in a broader context.

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Part A
Rat model

Chapter A1

Gene dosage effect on γ-secretase component Aph-1b in a rat model for neurodevelopmental disorders

With
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Abstract

A combination of genetic factors and early-life events is thought to determine the vulnerability of an individual to develop a complex neurodevelopmental disorder like schizophrenia. Pharmacogenetically selected, apomorphine-susceptible Wistar rats (APO-SUS) display a number of behavioural and pathophysiological features reminiscent of such disorders. Here we report microarray analyses revealing in APO-SUS rats, relative to their counterpart APO-UNSUS rats, a reduced expression of *Aph-1b*, a component of the γ-secretase enzyme complex that is involved in multiple (neuro)developmental signalling pathways. The reduced expression is due to a duplicon-based genomic rearrangement event resulting in an *Aph-1b* dosage imbalance. The expression levels of the other γ-secretase components were not different. However, γ-secretase cleavage activity was affected and the APO-SUS/-UNSUS *Aph-1b* genotypes segregated with a number of behavioural phenotypes. Thus, a subtle imbalance in the expression of a single, developmentally important protein may be sufficient to cause a complex phenotype.

Introduction

We used the susceptibility of normal outbred Wistar rats for the dopaminergic agonist apomorphine as a criterion for the selection of two distinct types of individuals (Cools et al., 1990; Ellenbroek and Cools, 2002). Through systemic apomorphine administration and long-term pharmacogenetic selections we produced rats with a high or low susceptibility for this drug (referred to as APO-SUS and APO-UNSUS rats, respectively). Extensive phenotyping of the APO-SUS and -UNSUS rats over the last 15 years has revealed differences in many aspects of behaviour, neuroanatomy, and their neurochemical, endocrine and immune systems (Cools et al., 1990; Ellenbroek and Cools, 2002). For example, they differ in brain information processing (prepulse inhibition and latent inhibition; (Ellenbroek et al., 1995)), locomotor activity in response to novelty, and fleeing and problem-solving behaviour (Cools, 1988; Cools et al., 1990). In addition, APO-SUS and -UNSUS rats show changes in their hypothalamus-pituitary-adrenal (HPA) axis response to stress (Rots et al., 1995), their sensitivity to dopamimetic drugs (Ellenbroek et al., 2000), their neuropeptide, steroid and steroid receptor levels (Cools et al., 1993; Rots et al., 1995; Rots et al., 1996), their synaptic densities within hypothalamic nuclei (Mulders et al., 1995), their T_{H2} cell contents and their susceptibilities to inflammatory and infectious diseases, vasorelaxation and stress (Kavelaars et al., 1997). Crossbreeding experiments have shown that genetic factors play an important role in the development of the rat model (Ellenbroek et al., 2000). Furthermore, the propensity of the model to develop the specific features in adulthood is dependent on the timing and type of stressors to which the rats have been exposed during early life (Ellenbroek et al., 2000; Degen et al., 2004). For example, when APO-SUS rats are reared from birth on by APO-UNSUS mothers their susceptibility to apomorphine is significantly reduced, whereas such crossfostering has no effect on APO-UNSUS rats. Conversely, a 24-h separation of the pups from their mother early in life enhances apomorphine susceptibility in APO-UNSUS rats, while this maternal deprivation does not affect APO-SUS rats (Ellenbroek et al., 2000). Interestingly, ten years after developing the original APO-SUS and -UNSUS lines a separate, independent selection and breeding procedure for apomorphine susceptibility of Wistar rats resulted in replication of the APO-SUS and -UNSUS lines that displayed similar features as the original ones (Ellenbroek et al., 2000). In this study, we examined the molecular basis of the differences between the APO-SUS and -UNSUS rats.

Materials and methods

Animals

Systemic administration of apomorphine (1.5 mg/kg s.c.) was used to select rats with a high or low susceptibility to this drug (APO-SUS and APO-UNSUS rats, respectively); the behaviour was quantified with the Ungerstedt box and the rats were classified on the basis of their gnawing scores (APO-SUS: >500 gnaws in 45 min; APO-UNSUS: <10 gnaws in 45 min) to select female and male rats for breeding the two distinct lines (Cools et al., 1990). We have avoided brother-sister pairings, and used a specific procedure that was aimed at selectively breeding for apomorphine susceptibility, while otherwise maintaining the genotypic heterogeneity of the initial Wistar strain. For the present study, we used APO-SUS and -UNSUS rats of the 13th to the 20th generation; genotyping of the original APO-SUS and -UNSUS lines was on rats of the 32nd generation. For the behavioural tests, adult male rats (PND60-70) were used, and separate I/I, II/II and III/III lines were generated by PCR-analysis of genomic DNA with specific primers and intermating each of the genotypes. The I/I and II/II sublines were produced from the APO-SUS line. Following its creation in 1994, the APO-SUS line has been maintained for 19 generations by random breeding. When we recently discovered the genomic recombination event reported here, we decided to select APO-SUS rats homozygous for one or two *Aph-1b* copies. The two rat populations were then intermated separately, resulting in the generation of the two APO-SUS sublines (I/I and II/II). Since no selection other than for *Aph-1b* homozygosity was applied, apart from the *Aph-1b* locus these sublines have highly similar general genetic backgrounds. Rats were bred and reared in the Central Animal Facility of the Radboud University under approved animal protocols and in accordance with institutional guidelines.

Microarray experiments

For mRNA expression profiling, hippocampi were dissected from fresh brains of PND 9 and PND 60 APO-SUS and -UNSUS rats and the tissues were frozen in liquid nitrogen. Furthermore, hippocampi were isolated from PND 60 APO-SUS and -UNSUS rats three hours after they were injected with apomorphine (1.5 mg/kg, s.c.). Total RNA was prepared with the Trizol reagent (Gibco BRL Life Technologies), and RNAs from 5 hippocampi were pooled and used in the microarray analyses. The PND 9 hippocampal samples were analysed using Affymetrix high-density oligonucleotide arrays (Rat Genome Set U34A containing ~7,000 full-length sequences and ~1,000 EST clusters randomly selected from *Rattus norvegicus* database sequences) according to the manufacturer's recommendations. Briefly, total RNA was purified on RNeasy columns (Qiagen) and 20 µg RNA was reverse transcribed (Superscript II; GibcoBRL Life Technologies) using a T7-dT24 primer (GenSet). Following second-strand synthesis, the double-stranded cDNA was used in an *in vitro* transcription

reaction to generate biotinylated cRNA. Purified, fragmented cRNA (10 µg) was hybridised onto the Affymetrix arrays for 16 h at 45 °C. Standard post-hybridisation washes and double-stain protocols were performed on a GeneChip Fluidics Station 400 (Affymetrix). Arrays were scanned on a Hewlett Packard Gene Array scanner and analysed using Microarray Suite version 5.0 software. For the PND 60 hippocampal samples, we used the Incyte high-density cDNA microarrays (Rat NeuroGEM 2.02 containing 8,478 Sprague Dawley rat nervous system cDNAs representing 6746 different genes). Isolation of mRNA, conversion to Cy3 or Cy5 fluorescently labelled cDNA, hybridisation and scanning were performed as described previously (Yue et al., 2001) and the data were analysed with GEMTools 2.5 software. All microarray experiments were performed with biological duplicates. With the oligonucleotide microarrays no differences were found that met the criteria (a difference in expression between PND 9 APO-SUS and -UNSUS hippocampus of >1.3-fold), while seven cDNAs were found to be differentially expressed on the cDNA arrays (i.e. >1.5-fold difference between PND 60 APO-SUS and -UNSUS hippocampus). However, the mRNA expression levels of four of these were not significantly different between APO-SUS and -UNSUS rats upon validation of the microarray data by quantitative RT-PCR. The differences in expression of two cDNAs (encoding transthyretin and prostaglandin D-synthase) were confirmed by the validation analyses but resulted from their expression in contaminating choroid plexus (dissection artefact during the isolation of the hippocampus). The *Aph-1b* transcript met the criteria and its ~1.6-fold reduction in hippocampal mRNA expression in basal and apomorphine-treated PND 60 APO-SUS relative to APO-UNSUS rats found on the cDNA microarrays was confirmed by quantitative RT-PCR (2.2 ± 0.3- and 3.1 ± 0.8-fold reduction in basal and apomorphine-treated PND 60 APO-SUS rats, respectively; n = 9). The *Aph-1b* transcript was not represented on the oligonucleotide microarrays.

Quantitative RT-PCR

For quantitative RT-PCR, first-strand cDNA was prepared from 2 µg of DNase I treated total RNA (isolated as described above) using Superscript II reverse transcriptase (Invitrogen). PCR samples contained 1X SYBR Green buffer, 3 mM $MgCl_2$, 0.4 mM dUTP and 0.2 mM each of dATP, dCTP and dGTP, 0.6 U AmpliTaq Gold (all from Applied Biosystems), 0.6 µM each oligonucleotide primer (Biolegio) and 1/20 synthesized cDNA in a 25-µl volume. Quantitative PCR was performed in a PE GeneAmp 5700 apparatus with conditions as follows: 10 min at 94 °C, then 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. *ß-Actin* was amplified from all samples to normalise expression. A control (no template) was included for each primer set. Data sets were analysed with Sequence Detection System 1.3 software. The following primers were used: *Aph-1b*-related (448-671):

5'-GTGATTCTCCTCAGTTCTTCCTTAATTC and 5'-GCCCATGAGCAC-CATGATTATAT; *Aph-1a* related (547-670): 5'-AGAGGAGACGGTACTGGGCTTT and 5'-ATGGAAACGGTGACTGCATAGA; *presenilin-1* (259-378): 5'-GTTCCTGTGACCCTCTGCATG and 5'-GCCTACAGTCTCGGTG-TCTTCTG; *presenilin-2* (1093-1218): 5'-GGAGACTTCATCTTCTACAGC-GTTCT and 5'-GAGCAGCAGGAGGGTGAGAC; *nicastrin* (504-622): 5'-TGGCTTGGCTTATGACGACTT and 5'-TCGGTGCAGAGCCATTCTG; *Pen-2* (14-162): 5'-GGGTGTCCAATGAGGAGAAGTT and 5'-TTGATTTGGCTCTGCT-CTGTGTA; *ß-actin* (346-435): 5'-CGTGAAAAGATGACCCAGATCA and 5'-AGAGGCATACAGGGACAACACA; numbers between brackets are nucleotide positions from start ATG). All PCR products were generated over intron-exon boundaries.

Genomic DNA analysis

APO-SUS and -UNSUS rats were genotyped by extensive Southern blot analysis and PCR screening of genomic tail DNA (primers and conditions available on request). Comparative analysis of the nucleotide sequences of rat *Aph-1b* and *–1b'* was performed with Vector NTI. The nucleotide sequences surrounding exons 5 and 5' of *Aph-1b* and *–b'*, respectively, and of the junction area within chimaeric *Aph-1b'/b* were determined by PCR analysis of genomic DNA from APO-UNSUS, and APO-SUS (II/II) and (I/I) rats using specific primers and subsequent nucleotide sequence analysis of the PCR products.

Northern blotting

Total RNA from hippocampus of PND 9 APO-UNSUS and APO-SUS (II/II), (II/I) and (I/I) rats, and from various tissues of PND 9 APO-UNSUS and APO-SUS (I/I) rats was isolated as described above, separated on gel (10 µg per lane), blotted and hybridised according to standard procedures with a full-length 798-bp rat *Aph-1b* cDNA probe detecting all *Aph-1b*-related mRNAs (~1,3 kb).

Western blotting

Protein extractions and immunoblottings were performed as previously described (Herreman et al., 2003). To examine Aph-1b protein expression, multiple tissue extraction methods and a variety of APO-SUS/-UNSUS and mouse tissues and cell lines were used. The polyclonal antibodies against presenilin-1 and nicastrin (Herreman et al., 2003), against presenilin-2 (Zymed Laboratories Inc.), and against Pen-2 and Aph-1a (Nyabi et al., 2003) were raised in rabbits. The antibodies against Aph-1b were directed against the peptides CLVRVITDNRDGPV and CVAGGSRRSL, and generated in rabbits (BioGenes GMBH, Germany). To examine

γ-secretase substrate cleavage, antibodies were used against the C-terminus of APP (C87, polyclonal antibody directed towards the most C-terminal 12 amino acids of APP and generated in rabbits), p75NTR (Mahadeo et al., 1994) and ErbB4 (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), and against β-tubulin (E7, (Chu and Klymkowsky, 1989) on brain tissue samples from three (I/I) rats of different nests and three (III/III) rats of different nests. Sample preparations for the analysis of APP, p75NTR and ErbB4 were performed as described by (Herreman et al., 2003), (Jung et al., 2003), and (Ni et al., 2001), respectively.

Behavioural analysis

For the apomorphine susceptibility test, rats were injected with 1.5 mg/kg apomorphine (s.c.) and their gnawing behaviour was tested in an Ungerstedt box (Cools et al., 1990). In the open field test, rats were placed in the centre of an elevated open field of 160 x 160 cm without walls. The open field was artificially subdivided into a central part (40 x 40 cm) and a peripheral area (16 cm in width). Locomotor behaviour was recorded for 30 min with a computerised automated tracking system, and the habituation time (defined as the time until the rat stopped locomotor activity for at least 90 s) was measured (Cools et al., 1990). The elevated plus maze consisted of a plexiglass four-armed maze with two open and two closed arms (10 x 50 cm). Each rat was placed in the centre of the plus maze facing a closed arm and the rat was allowed to explore the maze for 5 min.

Statistics

Data are presented as mean ± s.e.m. Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test where three groups were compared. For the comparison of two groups, the Student's t test was employed. Values of $P < 0.05$ were considered statistically significant.

Results

Gene expression profiling of APO-SUS and -UNSUS hippocampus

In an attempt to understand the difference between APO-SUS and -UNSUS rats at the molecular level, we decided to determine for both lines the mRNA expression profiles of the hippocampus of postnatal day 9 (PND 9) and PND 60 rats using oligonucleotide and cDNA microarrays. The hippocampus was selected because of its well-established physiological role in e.g. behavioural and HPA-axis regulation (McEwen, 2002), the neurochemical differences observed in APO-SUS and -UNSUS hippocampus (Ellenbroek and Cools, 2002), and the relative ease of its dissection. The time point PND 9 was chosen since exposing APO-UNSUS pups to a severe

stressor at this stage causes the most dramatic effect on brain information processing later on in life (Ellenbroek et al., 1998). At PND 60 the rats are just past their puberty and considered to be young adults, and at this age the clear phenotypic differences between APO-SUS and -UNSUS rats have been mapped (Ellenbroek et al., 2000; Cools and Ellenbroek, 2002; Ellenbroek and Cools, 2002). The mRNA expression profiling experiments revealed only one cDNA that met the preset criteria and could be confirmed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) (see Experimental Procedures for details). This transcript encodes the γ-secretase component Aph-1b, a predicted seven-transmembrane protein initially identified through genetic screens in worms (Francis et al., 2002; Goutte et al., 2002).

The Aph-1b gene in APO-SUS and -UNSUS rats

We next considered the possibility that the different *Aph-1b* mRNA expression levels in APO-SUS and -UNSUS were the result of a genomic DNA mutation. Database searches revealed that the rat (on chromosome 8q24) and mouse (on chromosome 9c) contain in tandem two *Aph-1b*-related copies (designated here *Aph-1b* and *Aph-1b'* with *-1b* downstream of *-1b'*), each consisting of six exons and spanning ~20 kb, and separated by ~24 kb. The single human *Aph-1b* gene (on chromosome 15q21.3) consists also of six exons, spans ~28 kb and represents the orthologue of rat/mouse *Aph-1b*. Computational approaches to define potential intron-exon structure, comparative (rat/mouse) nucleotide sequence analysis and EST database searches gave no indications for the presence of a gene in the intergenic region of *Aph-1b* and *-1b'*. Southern blot and PCR analyses of genomic DNA revealed that all APO-UNSUS tested (n = 93) contained three *Aph-1b*-related copies (here referred to as region III; Figure 1A), namely *Aph-1b'*, chimaeric *Aph-1b/b'* (consisting of exons 1-5 of *Aph-1b* and exon 6' of *Aph-1b'*) and *Aph–1b*. Of 151 APO-SUS genotyped, 26% were homozygous for the duplicated genes (II/II), 24% were homozygous for chimaeric *Aph-1b'/b* (consisting of exons 1'-5' of *Aph-1b'* and exon 6 of *Aph-1b*) (I/I), whereas the remaining 50% were heterozygous harbouring both the duplicated genes and chimaeric *Aph-1b'/b* (II/I) (Figure 1A). Interestingly, we found that the first-established APO-UNSUS and -SUS lines (Ellenbroek et al., 2000) displayed the same genotypes (i.e. all of the original APO-UNSUS tested were III/III, while all APO-SUS were II/II, II/I or I/I), indicating that the replication of the original APO-SUS/-UNSUS lines had resulted in the same *Aph-1b* genotypical distribution.

A comparative nucleotide sequence analysis of rat *Aph-1b* and *-1b'* showed a low degree of identity, except for the regions surrounding exons 5 and 5', and exons 6 and 6' (Figure 1B). A region of 1106 nucleotides containing exon 5/5' and identical between the two genes was found to represent the junction area of chimaeric *Aph-1b'/b* (Figure 1C). These results suggest that an unequal crossing-over (non-allelic

homologous recombination) between the identical regions around exons 5 and 5' (direct repeats) resulted in three in-tandem *Aph-1b*-related copies (region III) and chimaeric *Aph-1b'/b* (region I) (Figure 1D).

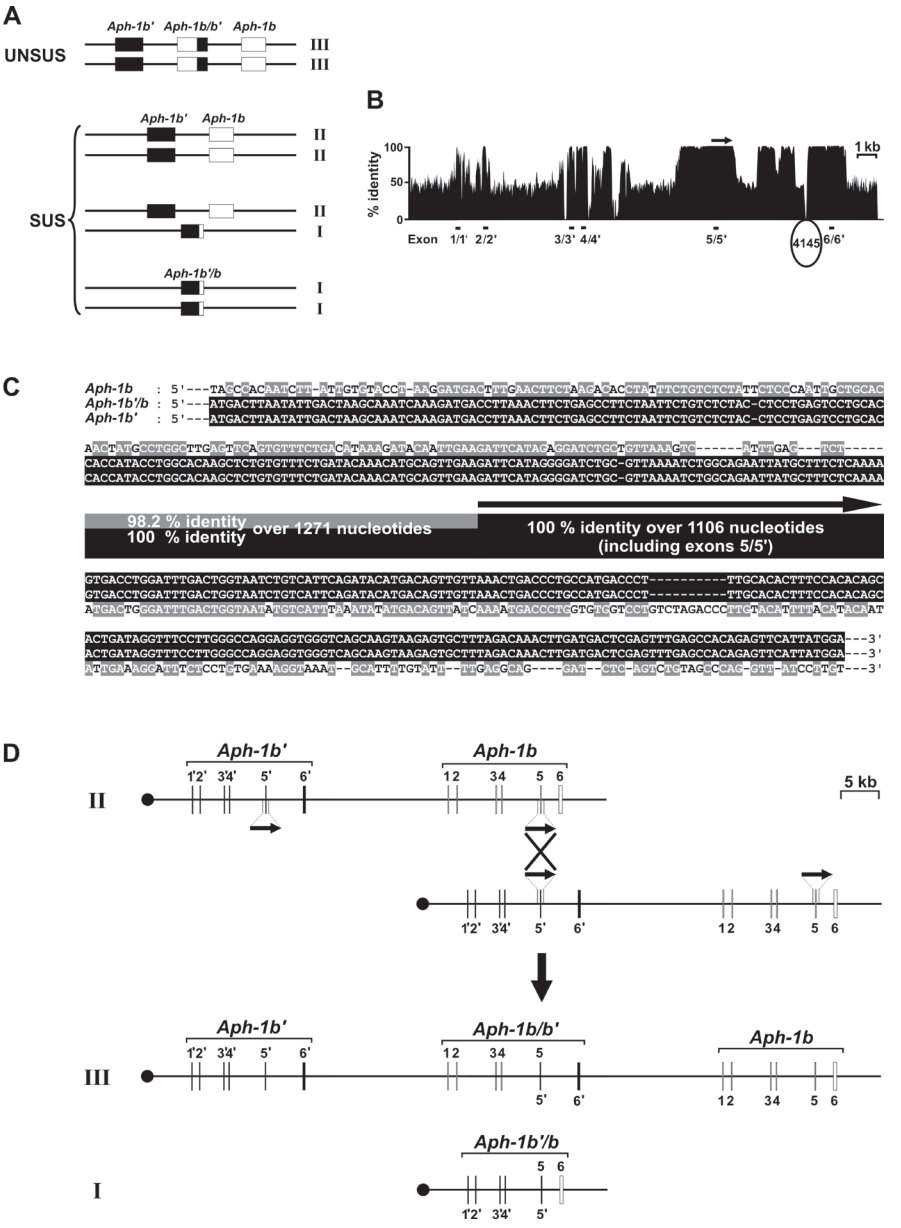
Expression of Aph-1b and the other γ-secretase components in APO-SUS and -UNSUS rats

Since a longitudinal study on hippocampus of postnatal stages of APO-SUS and -UNSUS rats (0, 2, 4, 6, 9, 12, 22, 35, 60 and 100 days of age) showed the largest differences in APO-SUS/-UNSUS *Aph-1b*-related mRNA expression at early post-natal stages (data not shown), rats of PND 9 were used for subsequent expression studies. Quantitative RT-PCR on hippocampus RNA revealed in PND 9 APO-SUS rats significantly lower levels of *Aph-1b*-related mRNAs than in PND 9 APO-UNSUS rats, namely a 2.3-, 3.2- and 6.2-fold reduction in APO-SUS (II/II), (II/I) and (I/I), respectively (Figure 2A). Northern blot analysis confirmed the quantitative RT-PCR data for hippocampus (Figure 2B) and furthermore showed, relative to PND 9 APO-UNSUS rats, clearly reduced levels of *Aph-1b*-related mRNA expression in all other PND 9 APO-SUS (I/I) tissues tested (Figure 2C). We were unable to reliably detect the Aph-1b protein, in line with the inability of others to detect this seven-transmembrane protein (Gu et al., 2003) and despite the fact that we generated two additional antibodies against two computationally selected and previously not chosen rat Aph-1b peptide regions.

Besides Aph-1b and its paralogue Aph-1a, the γ-secretase complex is presently thought to consist of three other physically interacting components, namely the putative enzymatic core multipass transmembrane protein presenilin-1 or -2, the type I integral membrane presenilin-associated glycoprotein nicastrin, and the small double-membrane-spanning protein Pen-2 (Fortini, 2002; Francis et al., 2002; Goutte et al., 2002). Quantitative RT-PCR revealed that in the hippocampus of PND 9 APO-UNSUS, and APO-SUS (II/II), (II/I) and (I/I) the mRNA levels of *Aph-1a*, *presenilin-1* and *-2*, *nicastrin* and *Pen-2* were not significantly different or only slightly affected (Figure 3A). Moreover, no significant differences in the protein levels of these γ-secretase components were observed in the hippocampus of PND 9 APO-UNSUS and -SUS rats (Figure 3B).

Cleavage activity of the γ-secretase enzyme in APO-SUS and -UNSUS rats

To examine the effect of the differential *Aph-1b* expression on γ-secretase enzyme activity, we performed western blot analysis of the cleavage products of the γ-secretase substrates amyloid-β precursor protein APP, p75 neurotrophin receptor (p75NTR) and neuregulin receptor ErbB4 in PND 2 APO-SUS (I/I) and APO-UNSUS (III/III) rat brain tissues (Figure 4). Since the ratios of the *Aph-1b* and *Aph-1a* mRNA levels


A
UNSUS
Aph-1b'
Aph-1b/b'
Aph-1b
III
III
SUS
II
II
II
I
Aph-1b'/b
I
I
B
% identity
100
50
0
1 kb
Exon 1/1' 2/2' 3/3' 4/4' 5/5' 4145 6/6'
C
Aph-1b
Aph-1b'/b
Aph-1b'
98.2 % identity over 1271 nucleotides
100 % identity
100 % identity over 1106 nucleotides
(including exons 5/5')
D
Aph-1b'
Aph-1b
II
5 kb
Aph-1b'
Aph-1b/b'
Aph-1b
III
Aph-1b'/b
I

greatly vary among rat tissues (data not shown), we decided to study tissues with a high *Aph-1b/-1a* mRNA ratio (pons/medulla, ratio ~ 3.8; olfactory bulb, ratio ~ 2.9) as well as tissues with a low ratio ([hypo]thalamus, ratio ~ 0.9; cerebellum, ratio ~ 0.7). The first substrate examined was the C-terminal fragment of APP (APP-CTF, also referred to as C83/C99), a well-defined direct γ-secretase substrate (De Strooper et al., 1998). No differences in the levels of APP-CTF were observed in the tissues with a low *Aph-1b/-1a* ratio (cerebellum and [hypo]thalamus), while a significant increase was detected in the olfactory bulb and pons/medulla (2.1- and 2.7-fold, respectively) of I/I compared to III/III rats. This finding indicates that γ-secretase cleavage activity was reduced in the APO-SUS (I/I) rats in tissues that normally express relatively high *Aph-1b* levels (olfactory bulb and pons/medulla). Cleavage by γ-secretase of the C-terminal fragment of p75NTR (p75NTR-CTF) yields the p75NTR intracellular domain (p75NTR-ICD; (Kanning et al., 2003). No major differences in the amounts of p75NTR-ICD were found in the cerebellum, (hypo) thalamus and pons/medulla, whereas in the I/I rats the levels of this product were significantly reduced in the olfactory bulb (1.7-fold). Similarly, the levels of the third γ-secretase substrate tested, the C-terminal fragment of ErbB4 (ErbB4-CTF), were significantly increased in the olfactory bulb of the I/I compared to the III/III rats (3.0-fold) and not in the other three tissues. Thus, the reduced expression of *Aph-1b* in the APO-SUS (I/I) rats has decreased γ-secretase cleavage activity in a tissue-dependent manner, i.e. only in tissues in which normally a high *Aph-1b/-1a* ratio occurs (pons/medulla and olfactory bulb) significant changes in activity could be detected.

Figure 1. *Aph-1b in APO-UNSUS and -SUS rats. (A) Schematic of the three Aph-1b-related copies in the APO-UNSUS rat (referred to as region III/III) and of the one or two gene copies in the APO-SUS rat (region II/II, II/I or I/I); black box, Aph-1b'; white box, Aph-1b; white/black box, chimaeric Aph-1b/b'; black/white box, chimaeric Aph-1b'/b. The results are based on Southern blot and PCR analyses of genomic DNA. (B) Schematic of the degree of nucleotide sequence identity between rat Aph-1b and -1b'. The locations of exons 1/1' to 6/6' of Aph-1b and -1b' are indicated by bars below the schematic. The loop indicates the absence of 4145 nucleotides in Aph-1b. The region of 1106 nucleotides identical between the two genes (surrounding exons 5 and 5', and representing the site of recombination; see under D) is indicated with an arrow above the schematic. (C) Alignment of the nucleotide sequences surrounding exon 5 of rat Aph-1b, exon 5/5' of chimaeric Aph-1b'/b and exon 5' of Aph-1b'. The 1106-bp region identical between the genes is indicated with an arrow (as in B and D). The 5'- and 3'-regions flanking the 1106-bp region in the chimaeric gene are identical to the corresponding regions in Aph-1b' and -1b, respectively. (D) Schematic of the genomic rearrangement resulting from unequal crossing-over (interchromosomal, non-allelic homologous recombination) between the in-tandem Aph-1b' and -1b (corresponding to region II in A) and leading to region III (Aph-1b', chimaeric Aph-1b/b' and Aph-1b), and region I (chimaeric Aph-1b'/b). The 1106-bp regions (direct repeat sequences) are depicted as arrows, recombination is shown by the X and dots are used for clarity in the presentation.*

Behavioural phenotypes of the I/I, II/II and III/III rats

We next wondered whether the I/I, II/II and III/III genotypes segregated with specific behavioural phenotypes and therefore performed a set of behavioural studies with adult rats of the three sublines. We first tested the susceptibility of the three lines for apomorphine by scoring their gnawing responses, and found that the III/III rats (32 ± 20 gnaws per 45 min) were significantly less susceptible for the drug than

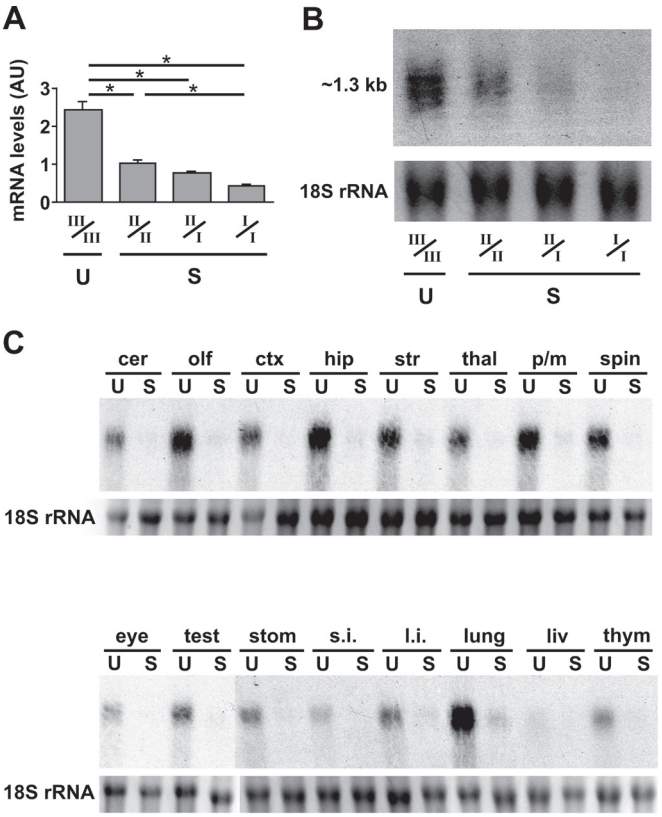


Figure 2. *Aph-1b mRNA expression in the hippocampus and other tissues of PND 9 APO-UNSUS (U) and APO-SUS (S) rats. (A) Quantitative RT-PCR on RNA extracted from hippocampus of PND 9 APO-UNSUS and APO-SUS (II/II), (II/I) and (I/I) rats. The primer sets detected all Aph-1b-related mRNAs. Results (n = 6 plus s.e.m.) were normalized towards β-actin and are expressed as arbitrary units (AU). Asterisks denote significant differences (P < 0.02). (B) Northern-blot analysis of RNA extracted from hippocampus of PND 9 APO-UNSUS and APO-SUS (II/II), (II/I) and (I/I) rats. The blot was hybridised with a full-length rat Aph-1b cDNA probe. As a control for RNA loading and integrity, 18S rRNA was used. (C) Northern blot analysis of RNA extracted from various tissues of PND 9 APO-UNSUS (III/III) and APO-SUS (I/I) rats. Tissues used were cerebellum (cer), olfactory bulb (olf), cortex (ctx), hippocampus (hip), striatum (str), (hypo)thalamus (thal), pons/medulla (p/m), spinal cord (spin), eye, testis (test), stomach (stom), small intestine (s.i.), large intestine (l.i.), lung, liver (liv) and thymus (thym). The blot was hybridised with a full-length rat Aph-1b cDNA probe. As a control for RNA loading and integrity, 18S rRNA was used.*

the II/II and I/I rats (1141 ± 189 and 1370 ± 99 gnaws per 45 min, respectively; Figure 5A). We then examined the explorative behaviour of the three sublines on a large open field. III/III rats habituated significantly faster (605 ± 76 s) than II/II and I/I rats, which were found to hardly habituate (1548 ± 98 s and 1536 ± 123 s, respectively; the maximum score was 1800 s) (Figure 5B, upper panel). For the III/III, II/II and I/I lines, a gradual increase in locomotor activity in the centre of the open field was found, with the I/I rats being significantly more active than the III/III rats (Figure 5B, lower panel), indicating a high explorative activity of the I/I rats in a cue-less environment. Finally, to assess novelty seeking in a stressful environment, the behaviours of the three sublines in the open *versus* the closed arms of the elevated plus maze were compared. The time spent on the open relative to the closed arms gradually increased for the III/III, II/II and I/I lines, with a significant difference between the III/III rats (8.0 ± 3.5) and the II/II and I/I rat lines (22.6 ± 5.0 and 30.5 ± 3.9, respectively) (Figure 5C, upper panel). The III/III rats travelled significantly shorter distances on the open relative to the closed arms than the II/II or I/I rats (Figure 5C, middle panel). Furthermore, the number of entries into the open *versus* the closed arms gradually increased for the III/III, II/II and I/I rats, and was significantly different between the III/III and I/I rats (Figure 5C, lower panel).

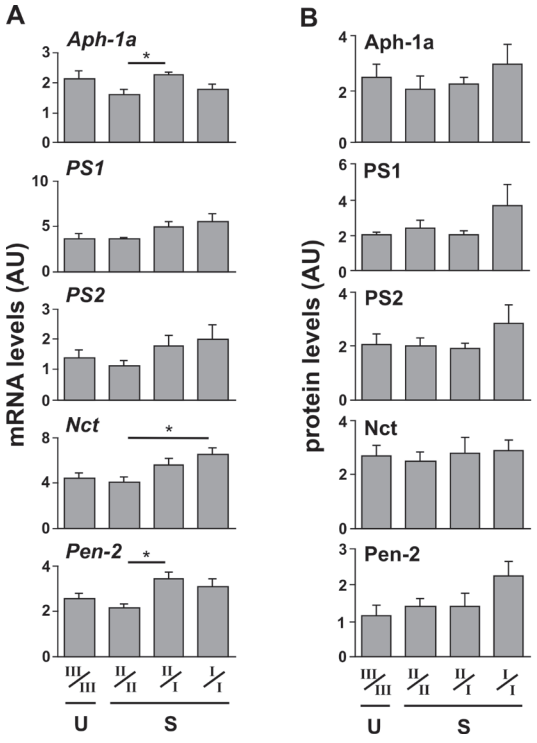


Figure 3. *mRNA and protein expression of γ-secretase components in the hippocampus of PND 9 APO-UNSUS (U) and -SUS (S) rats. (A) Quantitative RT-PCR analysis of Aph-1a, presenilin-1 (PS1) and -2 (PS2), nicastrin (Nct) and Pen-2 mRNAs in the hippocampus of PND 9 APO-UNSUS and APO-SUS (II/II), (II/I) and (I/I) rats. Results (n = 7 plus s.e.m.) were normalised towards β-actin and are expressed as arbitrary units (AU). Asterisks denote significant differences (P < 0.02). (B) Western blot analysis of the Aph-1a, PS1, PS2, Nct and Pen-2 proteins in the hippocampus of PND 9 APO-UNSUS and APO-SUS (II/II), (II/I) and (I/I) rats. Results (n = 6 plus s.e.m.) were normalised towards β-actin and are expressed as arbitrary units (AU).*

The results of the elevated plus maze therefore suggest that the I/I and II/II rats are less anxious and more active than the III/III rats. Together, the results of the apomorphine susceptibility, open field and elevated plus maze tests indicate that a number of behavioural phenotypes of the III/III, II/II and I/I rats segregated with the genotypes of the three sublines.

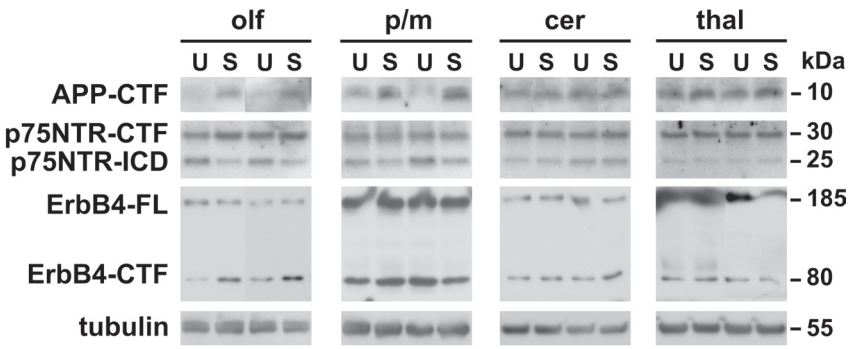


Figure 4. *Western blot analysis of the cleavage products of γ-secretase substrates in PND 2 APO-UNSUS (U, III/III) and APO-SUS (S, I/I) rat tissues. The cleavages of three direct γ-secretase substrates were investigated by analysing the levels of C-terminal fragments of amyloid-β precursor protein (APP-CTF), p75 neurotrophin receptor (p75NTR-CTF) and neuregulin receptor ErbB4 (ErbB4-CTF) in the olfactory bulb (olf), pons/medulla (p/m), cerebellum (cer), and (hypo)thalamus (thal) of APO-UNSUS (U, III/III) and APO-SUS (S, I/I) rats. Included for p75NTR is the analysis of its intracellular domain (p75NTR-ICD), a γ-secretase cleavage product. Tubulin was used for normalisation. Levels were significantly different between the I/I and III/III rats for APP-CTF, p75NTR-ICD and ErbB4-CTF in the olfactory bulb and for APP-CTF in the pons/medulla ($P < 0.05$; $n = 3$, with the three rats from different nests).*

Discussion

In this study, we explored the molecular genetic basis of APO-SUS rats that have a complex phenotype displaying a number of behavioural, neurochemical, endocrinological and immunological disturbances. Microarray analyses revealed the differential expression of only one gene (*Aph-1b*) that was found to be due to a gene-dosage effect with one or two *Aph-1b* copies in APO-SUS and three copies in APO-UNSUS rats. The dosage imbalance was caused by an unequal crossing-over event and the site of recombination was established, namely between direct repeats (a segmental duplication) within the *Aph-1b* locus in the rat genome. This recombination event is reminiscent of recently described human chromosomal rearrangements that involve segmental duplications, cause dosage imbalance of genetic material and

result in so-called genomic disorders; segmental duplications comprise at least 5% of the human genome and duplicon-based genomic rearrangements appear to occur *de novo* at a frequency of 0.7-1 in every 1000 births (Ji et al., 2000). However, in contrast to the situation in rodents, the human genome harbours only a single *Aph-1b.*

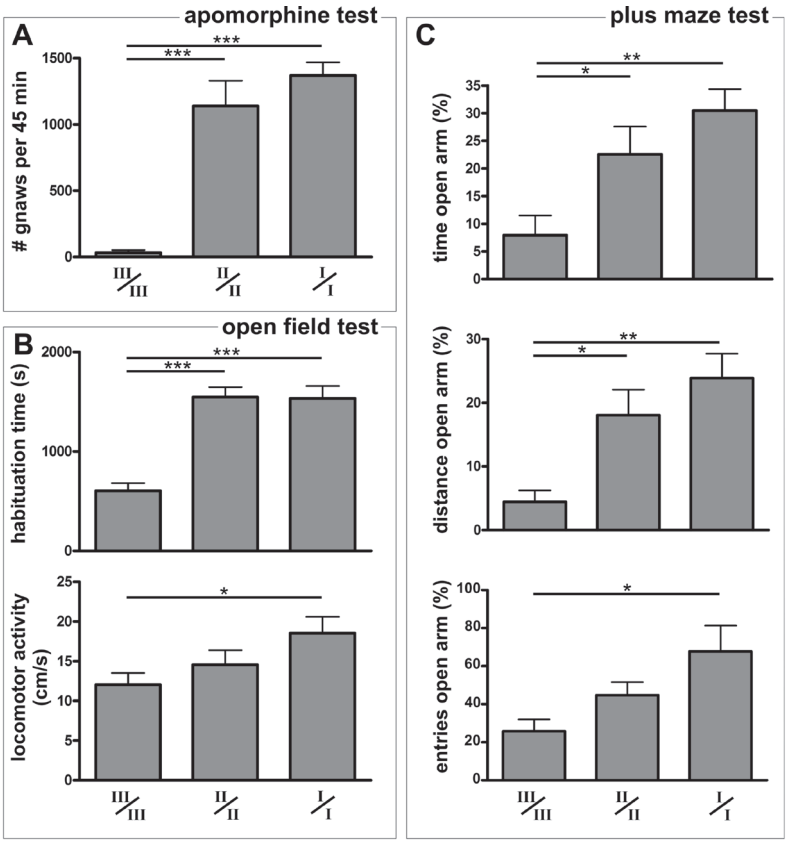


Figure 5. *Behavioural phenotypic analysis of adult rats with I/I, II/II and III/III genotypes. (A) Apomorphine susceptibility test for gnawing behaviour. Following 1.5 mg/kg apomorphine (s.c.) injection, the gnawing scores of III/III, II/II and I/I rats were recorded for 45 min (n = 12, 10 and 11, respectively; plus s.e.m.). (B) Open field test for explorative behaviour. III/III, II/II and I/I rats were analysed on a large open field for 30 min. Upper panel: the time period the rats have used to habituate (i.e. cease their locomotor activity for 90 s); lower panel: the locomotor activity in the centre of the open field (n = 11, 10 and 10, respectively; plus s.e.m.). (C) Elevated plus maze test for novelty seeking in a stressful environment. The walking patterns of III/III, II/II and I/I rats in the open and closed arms of the elevated plus maze were analysed for 10 min. Upper panel: the time spent in the open relative to the closed arms; middle panel: the relative distance travelled in the open relative to the closed arms; lower panel: the number of entries into the open relative to the closed arms (n = 11, 16 and 10 respectively; plus s.e.m.). Asterisks denote significant differences (*: P < 0.05; **: P < 0.01; ***: P < 0.001).*

In addition to the finding of its differential expression, a direct link between *Aph-1b* and the observed characteristics of the APO-SUS and -UNSUS rat lines is suggested by the fact that the replicated APO-SUS and -UNSUS lines displayed similar differences in behaviour and *Aph-1b* copy numbers as the original lines. Furthermore, the results of our behavioural tests indicated that the *Aph-1b* genotypes segregate with a number of behavioural APO-SUS and -UNSUS phenotypes. Aph-1b and its paralogue Aph-1a represent components of the γ-secretase enzyme complex and, together with Pen-2, are thought to be involved in the regulation of γ-secretase activity by modulating the biogenesis of presenilin-nicastrin complexes (De Strooper, 2003). We have indeed found that in the APO-SUS rats the lower *Aph-1b* levels had changed γ-secretase cleavage activity. The γ-secretase enzymatic machinery mediates intramembranous proteolytic cleavage of at least 14 type I transmembrane proteins that are thought to be involved in a complicated network of signalling pathways affecting many biological processes with a variety of physiological effects, especially during early (neuro)development (Fortini, 2002; De Strooper, 2003). Taken altogether, the results show that the reduced expression of *Aph-1b* underlies the APO-SUS phenotype. Thus, a subtle imbalance in the expression of a single gene product that is involved in a wide variety of developmental signalling pathways may well constitute the molecular basis of a complex phenotype that is generally believed to have a multifactorial background.

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Chapter A2

Ontogenic reduction of Aph-1b mRNA and γ-secretase activity in rats with a complex neurodevelopmental phenotype

With
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Abstract

Selectively bred apomorphine susceptible (APO-SUS) rats display a complex behavioural phenotype remarkably similar to that of human neurodevelopmental disorders, such as schizophrenia. We recently found that the APO-SUS rats have only one or two *Aph-1b* gene copies (I/I and II/II rats, respectively), whereas their phenotypic counterpart has three copies (III/III). Aph-1b is a component of the γ-secretase enzyme complex that is involved in multiple (neuro)developmental signalling pathways. Nevertheless, surprisingly little is known about γ-secretase expression during development. Here, we performed a longitudinal quantitative PCR study in embryos and the hippocampus of I/I, II/II and III/III rats, and found gene-dosage dependent differences in *Aph-1b*, but not *Aph-1a*, mRNA expression throughout pre- and post-natal development. On the basis of the developmental mRNA profiles, we assigned relative activities to the various *Aph-1a* and *-1b* gene promoters. Furthermore, in the three rat lines we observed both tissue-specific and temporal alterations in γ-secretase cleavage activity towards one of its best-known substrates, the amyloid-β precursor protein APP. We conclude that the low levels of *Aph-1b* mRNA and γ-secretase activity observed in the I/I and II/II rats during the entire developmental period may well underlie their complex phenotype.

Introduction

Selective breeding of Wistar rats that differ in their susceptibility to the dopamine receptor agonist apomorphine has resulted in the generation of two rat lines with either a high or a low susceptibility for this drug, the so-called APO-SUS and -UNSUS lines, respectively (Cools et al., 1990; Ellenbroek and Cools, 2002). Extensive phenotypic analysis of these lines revealed for the APO-SUS rats not only a strong, stereotyped gnawing response, but in addition many features that are also found in patients suffering from developmental psychiatric illnesses, such as schizophrenia. These features include information processing deficits in the brain (measured by a reduced prepulse inhibition and latent inhibition), a hyper-reactive dopaminergic pathway, an increased stress response, and a variety of behavioural, neurochemical, endocrinological and immunological features (Ellenbroek and Cools, 2002). While many of the differences between the APO-SUS and -UNSUS rat lines become apparent later in life, some features have been found at earlier developmental stages. For example, APO-SUS rats display a retarded development in comparison to APO-UNSUS rats (Degen et al., 2005), such as the development and maturation of the thymus and spleen (Cools et al., 1993). Retarded development (e.g. low birth weight and slower gestation) is also a hallmark of schizophrenia (Kunugi et al., 2001; Wahlbeck et al., 2001).

As the molecular basis of the differences between the APO-SUS and -UNSUS rats, we recently identified a gene-dosage imbalance of *Aph-1b* (Coolen et al., 2005b). APO-SUS rats have only one or two *Aph-1b* gene copies, whereas APO-UNSUS rats have three in tandem gene copies, resulting in reduced *Aph-1b* mRNA levels in the APO-SUS rats. The Aph-1b protein is a component of γ-secretase, an enzyme complex that regulates the intramembrane proteolysis of a number of type I membrane proteins, including Notch, neuregulin and the Alzheimer's disease-linked amyloid-β precursor protein APP (Kopan and Ilagan, 2004). These substrates play diverse physiological roles in multiple cell types and tissues, especially during early development.(Selkoe and Kopan, 2003) The minimal molecular subunit composition of an enzymatically active γ-secretase complex consists of presenilin (either PS-1 or PS-2), nicastrin (Nct), presenilin enhancer 2 (PEN-2) and the anterior pharynx defective 1 protein Aph-1, in mammals Aph-1aS, -1aL or -1b (Kimberly and Wolfe, 2003). We further established that the three *Aph-1b* rat genotypes segregated with a number of behavioural phenotypes (Coolen et al., 2005b). We now generated by crossbreeding, genetic reselection and phenotyping (susceptibility for apomorphine) three lines with one, two or three copies of the *Aph-1b* gene against an otherwise highly similar general genetic background (I/I, II/II and III/III lines, respectively). In this study, we performed an embryonic to adult longitudinal study on the mRNA

expression levels of the three Aph-1 family members in the I/I, II/II and III/III rat lines, and analysed the γ-secretase cleavage activity towards APP at a number of developmental stages.

Materials and methods

Animals

The generation of the APO-SUS and APO-UNSUS lines from Wistar rats with a high or low susceptibility to apomorphine, respectively, has been described previously (Cools et al., 1990). When we recently discovered the *Aph-1b* genotypes in the APO-SUS and -UNSUS rat lines (Coolen et al., 2005b), we decided to set up a crossbreeding scheme. Four male and four female I/I rats of the APO-SUS line were crossed with four female and four male III/III rats of the APO-UNSUS line, respectively. The offspring (either I/III or III/I) was intercrossed preventing brother-sister pairing and the resulting F_2 generation was genotyped for the *Aph-1b* locus by PCR analysis of genomic DNAs. The rats homozygous for either one or three *Aph-1b* gene copies were used to generate the I/I and III/III lines, respectively; apart from the *Aph-1b* locus, these lines have highly similar general genetic backgrounds. The crossbred I/I rats showed a significantly higher apomorphine susceptibility than the crossbred III/III rats. For the present studies, we used I/I and III/III rats of the F_3 generation of the crossbred lines. Similarly, the II/II rat line was generated by crossbreeding II/II rats of the APO-SUS line and III/III rats of the APO-UNSUS line. The presence of a vaginal plug was used to determine embryonic day 0 (ED0). Rats were bred and reared in the Central Animal Facility of the Radboud University under approved animal protocols and in accordance with institutional guidelines.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously (Coolen et al., 2005b). The following primers were used: *Aph-1b* (448-671): 5'-GTGATTCTCCTCAGTTCTTCC TTAATTC and 5'-GCCCATGAGCACCATGATTATAT; *Aph-1aL* (572-779):5'-CCTGGTAGT TGGGAGTCACCTT and 5'-CGCAGGGCAGAGTACACCAT; *Aph-1aS* (572-761): 5'-CCTGGTAGTTGGGAGTCACCTT and 5'-CGGTGCAGTCCAGGTAG TCAGT; *ß-actin* (346-435): 5'-CGTGAAAAGATGACCCAGATCA and 5'-AGAGGCATACAGGGACAACACA; numbers between brackets are nucleotide positions from start ATG. All PCR products were generated over intron-exon boundaries. PCR product analysis on a 2% agarose gel revealed a single band for each primer pair used. A control (no template) was included for each primer set. Data sets were analysed with Sequence Detection System 1.3 software.

Western blotting

Protein extractions and immunoblottings were performed as described (Herreman et al., 2003). The polyclonal antibody C87 directed towards the most C-terminal 12 amino acids of APP was used at a dilution of 1:3000, and detected both the APP full-length protein and the C-terminal fragment of APP (APP-CTF) (Coolen et al., 2005b). For quantification, the signals were analysed using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom). Western blot analysis of Aph-1b protein expression was not successful.

Statistics

Statistical evaluation of the quantitative RT-PCR data for the three Aph-1 family members was performed by means of a univariate analysis of variance (ANOVA) with as dependent variable the normalized transcript levels and as fixed factors the genotypes (I/I, II/II and III/III) and the developmental time points (either ED or PND). For every time point, data were further analyzed for significant differences between the three genotypes using a one-way ANOVA and where appropriate a post hoc Bonferroni test. Per genotype, the statistical analysis for differences in transcript levels during development was performed by means of an independent samples T-test to compare the two embryonic time points, or using a one-way ANOVA and a post hoc Bonferroni test for the postnatal time points. A probability of $P < 0.05$ was considered statistically significant. For the longitudinal Western blot analyses, the data sets were statistically analyzed by means of a univariate ANOVA with as dependent variable the APP-CTF/APP-FL levels and as fixed factors the genotypes and the developmental time points. Subsequent analysis using an independent samples T-test at every time point revealed significant differences between I/I and III/III rat tissues. Per rat line, a one-way ANOVA and where appropriate a post hoc Bonferroni analysis was used to identify differences between developmental time points. All statistical analyses were performed with the SPSS 12.0.1 software program (SPSS Inc., Chicago, Illinois, USA).

Results

Aph-1 mRNA levels in developing I/I, II/II and III/III rats

Real-time quantitative RT-PCR analysis of RNA from whole embryos (prenatal) and hippocampal tissue (postnatal) revealed similar overall *Aph-1b* mRNA expression profiles for the I/I, II/II and III/III rats with relatively low pre- and early postnatal expression that gradually increased over time until at ~PND 22 a plateau was reached (Figure 1A). Statistical analysis of the *Aph-1b* mRNA levels by means of a univariate ANOVA revealed significant differences between the genotypes, as well as

between the time points analysed (see supplemental tables 1-3 for statistical details). Subsequent one-way ANOVA per time point and post hoc Bonferroni analysis showed that the *Aph-1b* mRNA levels were significantly increased in the III/III hippocampal samples compared to the II/II or I/I samples at all pre- and postnatal time points tested, with the largest differences in the pre- and early postnatal samples (up to 16-fold reduction in ED13 embryos of I/I compared to III/III rats). Furthermore, in the postnatal II/II hippocampal samples the *Aph-1b* mRNA levels were higher than in the I/I samples and reached significant differences from PND 35 onwards (up to a two-fold increase). The mRNA expression patterns of *Aph-1aL* in the three rat lines were reminiscent of those of *Aph-1b* with significant increases from PND 35 onwards. In contrast, the developmental *Aph-1aS* mRNA expression levels were similar at all stages tested until PND 100, when a significant ~three-fold increase was observed (Figure 1C). For *Aph-1aL* as well as *Aph-1aS*, statistical analyses revealed no significant differences in mRNA expression levels between the three genotypes at any pre- or postnatal time point.

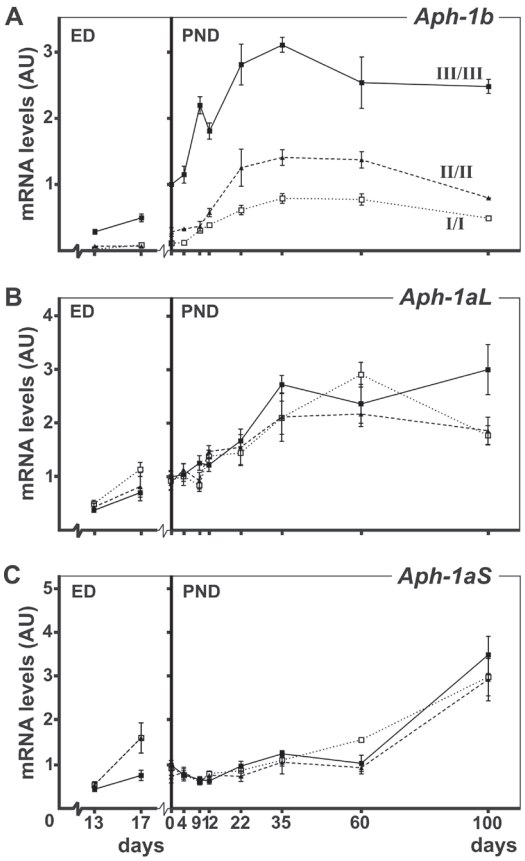

Figure 1. *Longitudinal study on Aph-1aL, -1aS and -1b mRNA expression levels in the I/I, II/II and III/III rats. Quantitative RT-PCR analysis of the mRNA levels of Aph-1b (A), Aph-1aL (B) and Aph-1aS (C) in total ED13 and ED17 embryos (left halves of the graphs) and in the hippocampus of PND 0, 4, 9, 12, 22, 35, 60 and 100 rats (right halves of the graphs). The Aph-1b primer set detected all Aph-1b gene transcripts; the Aph-1aL or Aph-1aS primer sets detected the long or short transcripts, respectively. Results were normalized towards β-actin mRNA levels and are expressed as arbitrary units (AU) with the level in the PND 0 III/III hippocampus set to 1 (per time point n = 3, plus s.e.m.). (A) Throughout development significant differences between the III/III and II/II rats as well as the III/III and I/I rats were observed for the Aph-1b mRNA levels ($P < 0.05$). From PND 35 onwards, Aph-1b mRNA levels also differed significantly between the II/II and I/I rats ($P < 0.05$). (B, C) No significant differences between the three genotypes were observed for the Aph-1aL or -1aS mRNA levels.*

γ-Secretase cleavage activity in developing I/I, II/II and III/III rats

Next, we were interested in the effects of the differential expression of *Aph-1b* mRNA in the I/I, II/II and III/III rats on γ-secretase cleavage activity. One of the best-known substrates of γ-secretase is the APP protein. The proteolytic processing of APP starts with shedding of its extracellular domain by α- or β-secretase, leaving a C-terminal fragment (CTF) that is subsequently cleaved by γ-secretase. Western blot analysis showed similar amounts of the APP holoprotein (APP-FL) in the olfactory bulb of PND 13 I/I, II/II and III/III rats, whereas the levels of the direct γ-secretase substrate APP-CTF were relatively high in the I/I, moderate in the II/II, and low in the III/III rats (Figure 2A). An increased level of APP-CTF implies reduced γ-secretase activity. Statistical analysis by means of a one-way ANOVA and post hoc Bonferroni revealed between the three genotypes a significant difference in the amounts of APP-CTF relative to APP-FL ($F[2,8] = 13.5$ $P < 0.05$), namely an ~1.4-fold and an ~1.8-fold higher ratio in the I/I rats relative to the II/II and III/III rats, respectively. Thus, the degree of proteolytic processing of APP by the γ-secretase complex correlated with the *Aph-1b* gene dosage in the I/I, II/II and III/III rats.

Since the *Aph-1b* mRNA expression levels significantly differed during development (Figure 1), we wondered whether the APP cleavage activity of γ-secretase would also show ontogenic dynamics. For this purpose, we first determined the γ-secretase cleavage activity (*i.e*. APP-CTF levels) in a number of tissues of I/I and III/III rats at PND 13 to identify appropriate tissues for a more detailed longitudinal analysis. Western blot analysis revealed variations in APP-CTF levels between the various tissues (Figure 2B). An accumulation of the APP-CTF levels was observed in the olfactory bulb, testis, spinal cord and lung of I/I compared to III/III rats and the latter two tissues were used for the more detailed study. In the spinal cord, the levels of APP-FL gradually decreased over time for both I/I and III/III rats from PND 9 onwards, whereas the APP-CTF levels increased. In contrast, lung tissue showed similar expression levels of the holoprotein throughout postnatal development, while the APP-CTF levels were markedly increased in both I/I and III/III rats from PND 13 onwards (Figure 2C and D). Statistical analysis of the APP-CTF/APP-FL ratio per time point revealed a significant increase of APP-CTF levels in the spinal cord of I/I compared to III/III rats already at PND 2 and also at PND 13 and 35, whereas in the lung significant accumulations were found from PND 13 onwards (see also supplemental table 4 for statistical details). Thus, during development of the I/I, II/II and III/III rats the differential *Aph-1b* expression levels have spatio-temporal effects on γ-secretase cleavage activity towards APP.

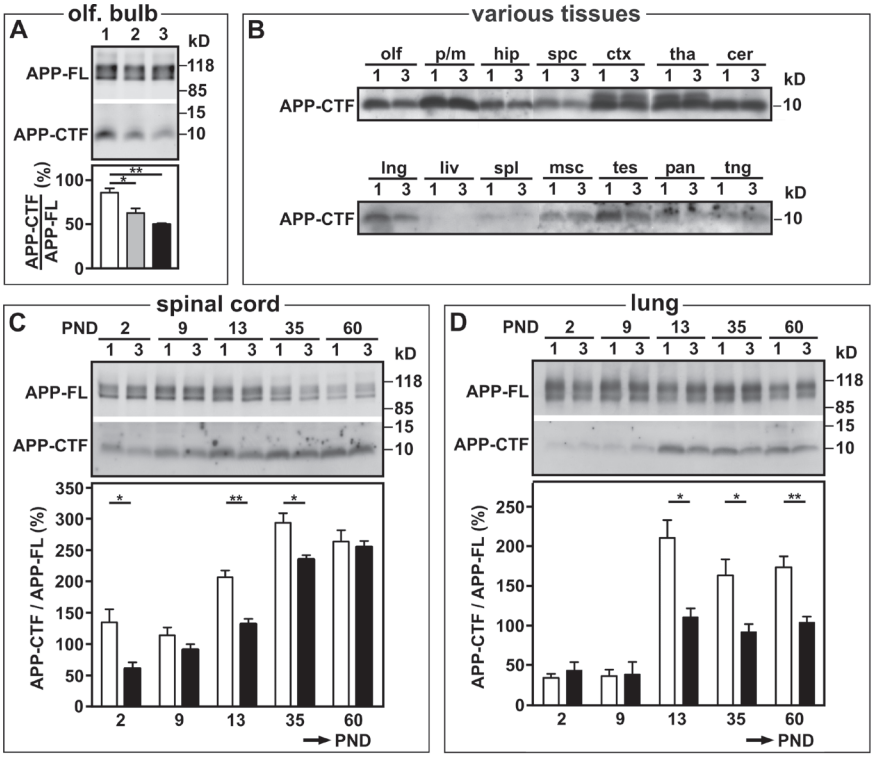

Figure 2. *Ontogenic analysis of γ-secretase cleavage activity towards APP in the I/I, II/II and III/III rats. (A) APP processing by γ-secretase is dependent on the number of Aph-1b gene copies. Western blot analysis of the levels of the APP holoprotein (APP-FL) and C-terminal fragment (APP-CTF) was performed in the olfactory bulb of I/I (1), II/II (2) and III/III (3) rats at PND 13. Quantification of the APP-CTF relative to the APP-FL levels revealed significant differences between the I/I and II/II, and between the I/I and III/III rats (*: P < 0.05; **: P < 0.02; n = 3, with the three rats from different nests, plus s.e.m.). (B) Western blot analysis of the APP-CTF levels in a number of tissues of PND 13 I/I (1) and III/III (3) rats. Tissues analysed were olfactory bulb (olf), pons/medulla (p/m), hippocampus (hip), spinal cord (spc), cortex (ctx), (hypo)thalamus (tha), cerebellum (cer), lung (lng), liver (liv), spleen (spl), muscle (msc), testis (tes), pancreas (pan) and tongue (tng). The additional product observed in cortex and (hypo)thalamus corresponds to the β-secretase cleavage product CTFβ (C99). (C, D) Western blot analysis of the levels of APP-FL and -CTF in the spinal cord (C) and lung (D) of I/I (1) and III/III (3) rats at PND 2, 9, 13, 35 and 60. Significantly elevated APP-CTF/-FL ratios in I/I compared to III/III rats were found in the spinal cord at PND 2, 13 and 35 and in the lung from PND 13 onwards. (*: P < 0.05; **: P < 0.02; n = 3, with the three rats per genotype from different nests, plus s.e.m.).*

Discussion

The γ-secretase enzyme is involved in a large variety of developmental signalling pathways (Artavanis-Tsakonas et al., 1999; Huang et al., 2000; Turner et al., 2003;

Kopan and Ilagan, 2004). It is therefore surprising that little is known about the developmental expression patterns of PS, Nct, PEN-2 and Aph-1, the components of the γ-secretase complex. In the present study, we examined the mRNA expression levels of the three Aph-1 family members (*Aph-1b*, *-1aS* and *-1aL*) and the γ-secretase cleavage activity towards APP during the development of rats with one, two or three *Aph-1b* gene copies (I/I, II/II and III/III rats, respectively). Relatively low levels of *Aph-1b* mRNA were detected during the pre- and early postnatal developmental stages, whereas from PND 35 onwards we found increased levels that remained high during further development. This developmental *Aph-1b* mRNA expression profile thus suggests that in the rat the demand for Aph-1b is higher postnatally than during early development. The availability of the three *Aph-1b* mRNA expression profiles together with the knowledge of the genetic make-up of the *Aph-1b* loci in the I/I, II/II and III/III rat lines allows us to speculate about the developmental activity of the various *Aph-1b* gene promoters; the different sets of *Aph-1b* genes and gene promoters in the three rat lines are schematically depicted in Figure 3. Despite the extra gene copy in the II/II rats, the *Aph-1b* mRNA expression levels were not different during early development of the I/I and II/II rats, indicating that prenatally either the *Aph-1b* gene promoter displays a relatively low activity or the *Aph-1b'/b* hybrid gene promoter has a relative high activity. Conversely, in the III/III rats we found higher *Aph-1b* mRNA expression levels than one would expect on the basis of three gene copies and the promoter of the *Aph-1b/-1b'* hybrid gene thus appears to display a relatively high activity throughout development. The observed *Aph-1b* mRNA expression levels are therefore not always in full accordance with the number of *Aph-1b* gene copies.

Neither pre- nor postnatally the levels of *Aph-1aS* and *-1aL* mRNA differed between the three rat lines, indicating that these paralogues did not compensate for the altered *Aph-1b* mRNA levels. This is in line with the results of recent RNA interference and knockout studies on the *Aph-1* family members in the mouse showing that the expression levels of the other γ-secretase components were affected only when *Aph-1a* expression was silenced and not with abolished *Aph-1b* expression (Shirotani et al., 2004; Saito and Araki, 2005; Serneels et al., 2005). Similar to the results obtained with reduced *Aph-1a* expression, knock down or knock out of either of the γ-secretase components PS1, Nct or PEN-2 in the mouse led to affected levels of most of the nonsilenced γ-secretase subunits (Chen et al., 2003; Gu et al., 2003; Li et al., 2003a; Li et al., 2003b; Takasugi et al., 2003; Hasegawa et al., 2004; Zhang et al., 2005). In contrast, ablation of mouse *PS2* expression had little effect on the expression levels of the other γ-secretase components (Herreman et al., 1999; Chen et al., 2003; Zhang et al., 2005), comparable to what we have observed in the I/I, II/II and III/III rat lines concerning the effect of reduced *Aph-1b* expression (Coolen et

al., 2005b). These findings suggest that PS2 and Aph-1b are somehow related.
The reduced ontogenic *Aph-1b* mRNA levels observed in the hippocampus may well underlie the large differences in stress susceptibility of the rats with one, two or three Aph-1b gene copies (Coolen et al., 2005a), as the hippocampus is known to modulate the stress axis (Knigge, 1961; Jacobson and Sapolsky, 1991). In addition, the marked hippocampal differences may be related to the dopaminergic hyperreactivity of the rats, since one of the most important projections of the hippocampus runs to the ventral striatum (Groenewegen et al., 1999). In psychiatric disorders, such as schizophrenia, stress susceptibility and dopaminergic hyperreactivity have also been observed (Lammers et al., 1995; Muller-Spahn et al., 1998). According to the developmental hypothesis of schizophrenia, the pathophysiology and aetiology of the disorder are related to an affected development or maturation of the brain. Epidemiological studies have provided a solid basis for this hypothesis, e.g. during early life schizophrenic individuals have shown a retarded motor and cognitive development (Jones, 1997; Isohanni et al., 2000; Cannon et al., 2002). Furthermore, disturbances in the cytoarchitecture of the hippocampal formation (Kovelman and Scheibel, 1984) and entorhinal cortex (Bernstein et al., 1998) can only be adequately explained by aberrant brain development. Unfortunately, schizophrenia has an adult onset and it has turned out to be difficult to elucidate its molecular basis (Andreasen, 2000). Perhaps the results of our longitudinal developmental study on the I/I, II/II and III/III rat lines may help in the understanding of the molecular background of such neurodevelopmental disorders.

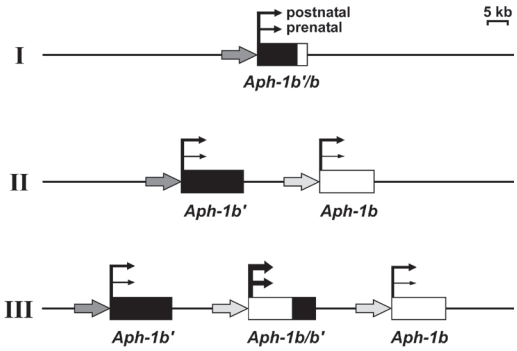

Figure 3. *Schematic representation of the Aph-1b genes in the I/I, II/II and III/III rat lines. The I/I rats have only one Aph-1b gene (the Aph-1b'/-1b hybrid gene consisting of exons 1-5 of Aph-1b' and exons 5-6 of Aph-1b), the II/II rats contain the Aph-1b' and Aph-1b genes, and the III/III rats harbor, besides the Aph-1b' and Aph-1b genes, an additional Aph-1b/-1b' hybrid gene . The activities of the various promoters, as deduced from the developmental mRNA expression profiles (Figure 1), are indicated by the black arrows with their thickness corresponding to the level of activity (lower arrows: prenatal activity; upper arrows: postnatal activity). Open arrows indicate the promoters of the various genes.*

We have found that in the three rat lines the *Aph-1b* mRNA expression levels correlated with the degree of APP processing by γ-secretase. Similarly, complete silencing of the expression of *PS1, Nct, PEN-2* or *Aph-1a* in the mouse resulted in reduced γ-secretase activity (also indicated by elevated APP-CTF levels) (Rozmahel et al., 2002; Li et al., 2003a; Hasegawa et al., 2004; Ma et al., 2005; Serneels et al., 2005; Zhang et al., 2005). Conversely, preventing mouse *PS2* or *Aph-1b* expression did not affect or only slightly decreased γ-secretase activity, respectively, and gave only a mild phenotype, whereas removal of *PS1*, *Nct* or *Aph-1a* is lethal (Herreman et al., 1999; Serneels et al., 2005). We therefore hypothesize that the functioning of the PS2- and Aph-1b-containing γ-secretase complex is different from that of the complexes with other subunit compositions.

The reduction in APP processing that we observed may be related to the hyperactive behaviour displayed by rats with one or two *Aph-1b* gene copies (Coolen et al., 2005b). Interestingly, in mice the reverse situation, namely overexpressed APP (or APP-CTF), results in general hypoactive behaviour (D'Hooge et al., 1996; Lalonde et al., 2002). We further found that the effects on γ-secretase activity were tissue specific. In general, in a tissue with a high level of *Aph-1b* mRNA compared to the *Aph-1a* mRNA level (a high *Aph-1b/-1a* mRNA ratio) relatively large differences in the APP-CTF levels were found between the I/I and III/III rats. In the hippocampus, a tissue with a relatively low Aph-1b/-1a ratio (Coolen et al., 2005a), we did not observe significant differences in γ-secretase cleavage activity between the three rat lines, while large alterations in hippocampal *Aph-1b* mRNA levels were detected (~8-fold at PND 13). It thus appears that a reduced *Aph-1b* expression causes a more severe effect on γ-secretase activity in tissues with a high Aph-1b/-1a ratio than in tissues with a low ratio. Furthermore, sufficient amounts of the direct γ-secretase substrate APP-CTF had to be present in a tissue to allow detection of any significant difference in γ-secretase cleavage activity. For instance, the lung has a high Aph-1b/-1a ratio, but we did not observe an affected γ-secretase activity in early postnatal lung, presumably due to the low levels of APP-CTF in this tissue.

The γ-secretase complex is able to cleave an ever-growing list of now at least 15 substrates and it is likely that in the I/I rats the effect of the reduced *Aph-1b* mRNA expression was not restricted to the decreases in APP cleavage, but that the cleavages of other (developmentally important) γ-secretase substrates, like Notch, neuregulin, ErbB4 and N-cadherin, were also affected. Such a broad ontogenic effect presumably resulted in not only retarded development (Cools et al., 1993; Degen et al., 2005), but also in the complex phenotype of the SUS rats in adulthood (Ellenbroek and Cools, 2002; Coolen et al., 2005b).

In conclusion, a subtle ontogenic imbalance in the expression of a single γ-secretase component causes spatio-temporal differences in γ-secretase enzymatic activity.

Because γ-secretase complexes with different subunit compositions are not functionally redundant and each complex is involved in the preferential cleavage of a subset of γ-secretase substrates (Hebert et al., 2004; Shirotani et al., 2004; Coolen et al., 2005b), a complex (neuro)developmental phenotype may arise in an organism with altered expression of a γ-secretase component. Thus, affecting the ontogenic expression of a single developmentally important protein may ultimately result in a complex phenotype later in life.

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Supplemental tables

Supplemental table 1. Statistical support for Figure 1 by means of a univariate ANOVA on longitudinal mRNA expression levels of Aph-1 family members in the hippocampus of I/I, II/II and III/III rats (significant if $P < 0.05$; NS: no significant differences).

	Aph-1b	Aph-1aS	Aph-1aL
ED	geno: F(2,19) = 57.6 ED: F(1,19) = 9.8 geno*ED: F(2,19) = 4.5	geno: NS ED: F(1,21) = 12.6 geno*ED: NS	geno: NS ED: F(1,22) = 18.9 geno*ED: NS
PND	geno: F(2,81) = 242.3 PND: F(1,81) = 26.8 geno*PND F(1,81) = 3.2	geno: NS PND: F(1,86) = 64.1 geno*PND: NS	geno: NS PND: F(1,86) = 20.0 geno*PND: NS

Supplemental table 2. Since the univariate ANOVA for Figure 1 yielded statistical significance, we performed for the I/I, II/II and III/III rats over the embryonic time points an independent samples T-test and over the postnatal time points a one-way ANOVA and where appropriate a post hoc Bonferroni analysis. Significant differences between time frames are indicated (significant if $P < 0.05$; NS: no significant differences).

		ED	PND	
		independent samples T-test	**one-way ANOVA**	**Bonferroni**
Aph-1b	I/I	NS	PND: F(7,27) = 17.7	PND(0-4)<PND(22-100) PND(9-12) < PND(35-60)
	II/II	NS	PND: F(7,25) = 5.5	PND(0) < PND(22)
	III/III	ED13 < ED17	PND: F(7,26) = 17.2	PND(0-4)<PND(22-100) PND(0-4) < PND(9) PND(12) < PND(22-35)
Aph-1aS	I/I	NS	PND: F(7,27) = 19.1	PND0-60) < PND(100)
	II/II	NS	PND: F(7,27) = 17.9	PND(0-60) < PND(100)
	III/III	NS	PND: F(7,29) = 29.8	PND(0-60) < PND(100)
Aph-1aL	I/I	NS	PND: F(7,27) = 11.6	PND(0-9) < PND(35-60)
	II/II	NS	PND: F(7,27) = 3.2	NS
	III/III	NS	PND: F(7,29) = 11.4	PND(0-12) < PND(35-100)

Supplemental table 3. Since the univariate ANOVA for Figure 1 yielded statistical significance, we performed for every developmental time point a comparison of the transcript levels of the I/I, II/II and III/III rats by means of a one-way ANOVA and where appropriate a post hoc Bonferroni analysis. Significant differences between genotypes are indicated (significant if $P < 0.05$; NS: no significant differences).

			Aph-1b	Aph-1aS	Aph-1aL
ED	**13**	one-way ANOVA	geno: F(2,8) = 34.1	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	17	one-way ANOVA	geno: F(2,9) = 34.0	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
PND	**0**	one-way ANOVA	geno: F(2,8) = 129.5	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	4	one-way ANOVA	geno: F(2,8) = 52.0	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	9	one-way ANOVA	geno: F(2,8) = 148.2	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	12	one-way ANOVA	geno: F(2,8) = 90.9	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	22	one-way ANOVA	geno: F(2,10) = 10.7	NS	NS
		Bonferroni	(I/I or II/II) < III/III	-	-
	35	one-way ANOVA	geno: F(2,8) = 135.5	NS	NS
		Bonferroni	I/I < II/II < III/III	-	-
	60	one-way ANOVA	geno: F(2,15) = 51.9	NS	NS
		Bonferroni	I/I < II/II < III/III	-	-
	100	one-way ANOVA	geno: F(2,8) = 295.3	NS	geno: F(2,8) = 5.2
		Bonferroni	I/I < II/II < III/III	-	NS

Supplemental table 4. Statistical support for Figure 2 by means of a univariate ANOVA on longitudinal APP-CTF levels in the spinal cord and lung of I/I and III/III rats. Since the univariate ANOVA yielded statistical significance, we performed an independent samples T-test over the APP-CTF levels for every developmental time point, and for both I/I and III/III rats a one-way ANOVA and post hoc a Bonferroni analysis. Significant differences between genotypes or time frames are indicated (significant if $P < 0.05$; NS: no significant differences).

spinal cord

univariate ANOVA
geno: NS
PND: F(4,30) = 15.6
geno*PND: NS

PND	independent samples T-test
2	NS
9	NS
13	I/I > III/III
35	I/I > III/III
60	NS

geno	one-way ANOVA	Bonferroni
I/I	F(4,14) = 4.4	PND(2) < PND(35)
III/III	F(4,14) = 46.2	PND(2-13) < PND35-60)

lung

univariate ANOVA
geno: F(1,30) = 13.1
PND: F(4,30) = 22.7
geno*PND: F(4,30) = 3.4

PND	independent samples T-test
2	NS
9	NS
13	NS
35	I/I > III/III
60	I/I > III/III

geno	one-way ANOVA	Bonferroni
I/I	F(4,14) = 21.3	PND(2-9) < PND(13-60)
III/III	F(4,14) = 4.4	NS

Chapter A3

Reduced Aph-1b expression causes tissue- and substrate-specific changes in γ-secretase activity in rats with a complex phenotype

With
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Abstract

The γ-secretase enzyme complex displays intramembrane catalytic activity towards many type I transmembrane proteins, including the Alzheimer-linked amyloid β-protein precursor (APP) and the neuregulin receptor ErbB4. Active γ-secretase is a tetrameric protein complex consisting of presenilin-1 (or -2), nicastrin, PEN-2, and Aph-1a (or -1b). We have recently discovered that pharmacogenetically bred apomorphine-susceptible Wistar rats (APO-SUS) have only one or two copies of the *Aph-1b* gene (termed I/I and II/II rats, respectively), whereas their phenotypic counterparts (APO-UNSUS) have three copies (III/III). As a result, APO-SUS rats display reduced *Aph-1b* expression and a complex phenotype reminiscent of neurodevelopmental disorders. Here we determined in the I/I and III/III rats the γ-secretase cleavage activity towards the three APP superfamily members, p75 neurotrophin receptor, ErbB4 and neuregulin-2, and found that the cleavage of only a subset of the substrates was changed. Furthermore, the observed differences were restricted to tissues that normally express relatively high *Aph-1b* compared to *Aph-1a* levels. Thus, we provide *in vivo* evidence that subtle alterations in γ-secretase subunit composition may lead to a variety of affected (neuro)developmental signalling pathways and, consequently, a complex phenotype.

Introduction

The γ-secretase complex belongs to the family of aspartyl proteases and cleaves many type I transmembrane proteins within their membrane domain, after removal of the ectodomain (Kopan and Ilagan, 2004). The complex is notoriously known because of its role in the pathological production of amyloid-β in Alzheimer's disease (reviewed in Fortini, 2002; Sisodia and St George-Hyslop, 2002; Tanzi and Bertram, 2005). Under normal physiological conditions, γ-secretase causes the release of the intracellular domain (ICD) of a growing list of proteins, such as the amyloid β-precursor protein (APP), its relatives the APP-like proteins APLP1 and APLP2, Notch, neuregulin, the neuregulin receptor ErbB4, p75 neurotrophin receptor, N-cadherin and ApoER2. These type I transmembrane proteins are part of multiple (neuro)developmental signalling pathways (reviewed in Koo and Kopan, 2004; Kopan and Ilagan, 2004). Recently, the minimal molecular subunit composition of the γ-secretase complex has been solved, namely presenilin (either PS-1 or PS-2), nicastrin (Nct), presenilin enhancer 2 (PEN-2) and the anterior pharynx defective 1 protein Aph-1, in mammals Aph-1aS, -1aL or -1b (De Strooper et al., 1998; Yu et al., 2000; Francis et al., 2002; Edbauer et al., 2003). In total six complexes with different subunit compositions can be formed because of the two presenilin proteins and the three Aph-1 proteins (Shirotani et al., 2004). It has been widely accepted that PS is the catalytic core protein of the complex, but the specific functions of the other γ-secretase components are less clear. Nct may have a role in stabilizing the complex or create a substrate docking site of the complex (Berezovska et al., 2003; Zhang et al., 2005), while Aph-1 has been suggested to stabilize both the maturing and final γ-secretase complex (Lee et al., 2004), and PEN-2 may assist in the endoproteolysis of the presenilin holoprotein during final maturation of the complex (reviewed in Periz and Fortini, 2004).

In a previous gene expression profiling study, we discovered a reduced expression of *Aph-1b* mRNA as the only difference between a pharmacogenetically selected apomorphine-susceptible (APO-SUS) rat line displaying many features of a complex neurodevelopmental disorder and its phenotypic counterpart, the apomorphine-unsusceptible (APO-UNSUS) line (Coolen et al., 2005). The APO-SUS and -UNSUS rats differ not only in information processing deficits in the brain (measured by pre-pulse inhibition and latent inhibition), but also show hyperactivity in an open field and in the elevated-plus maze, a hyper-reactive dopaminergic pathway, an increased stress response, and a variety of behavioural, neurochemical, endocrinological and immunological features (Ellenbroek and Cools, 2002). A detailed genomic analysis of the rat lines revealed an imbalance in *Aph-1b* gene copy numbers; APO-SUS rats have only one or two copies of the gene, whereas the APO-UNSUS genome

contains three in-tandem gene copies (Coolen et al., 2005). We now generated via cross-breeding and genetic re-selection two new rat lines; one homozygous for the allele with a single *Aph-1b* gene (the I/I rat line) and one homozygous for the allele with three gene copies (the III/III rat line). Here, we analysed in various tissues of the I/I and III/III rats the γ-secretase cleavage activity by determining the endogenous levels of protein fragments derived from a number of γ-secretase substrates, namely the three APP superfamily members, p75, ErbB4 and neuregulin-2 (NRG2).

Materials and methods

Animals

Initially, systemic administration of apomorphine (1.5 mg/kg s.c.) was used to select Wistar rats with a high or low susceptibility to this drug (APO-SUS and APO-UNSUS rats, respectively). The evoked stereotyped gnawing behaviour (APO-SUS: >500 gnaws in 45 min; APO-UNSUS: <10 gnaws in 45 min) was used to select female and male rats for breeding the two distinct lines (Cools et al., 1990). Phenotyping of the rat lines was reviewed elsewhere (Ellenbroek and Cools, 2002). When we recently discovered the different *Aph-1b* genotypes in the rat lines (Coolen et al., 2005), we decided to use rats of the 21st generation to set up a crossbreeding scheme. Four male and four female I/I rats of the APO-SUS line were crossed with four female and four male III/III rats of the APO-UNSUS line, respectively. The offspring (either I/III or III/I) was inter-crossed preventing brother-sister pairing and the resulting F_2 generation was genotyped for the *Aph-1b* locus by PCR analysis of genomic DNAs. The rats homozygous for either one or three *Aph-1b* gene copies were used to generate I/I and III/III lines, respectively; apart from the *Aph-1b* locus, these lines have highly similar general genetic backgrounds. The crossbred I/I rats showed a significantly higher apomorphine susceptibility than the crossbred III/III rats. For the present γ-secretase cleavage activity studies, we used I/I and III/III rats of the F_3 generation of the crossbred lines. Rats were bred and reared in the Central Animal Facility of the Radboud University under approved animal protocols and in accordance with institutional guidelines.

Northern blot analysis

Total RNA from various tissues of PND 9 I/I and III/III rats was isolated with the Trizol reagent (Gibco BRL Life Technologies), separated on gel (10 μg per lane), blotted and hybridised according to standard procedures with a full-length 798-bp rat *Aph-1b* cDNA probe detecting one product of ~1.3 kb. After stripping, the blot was rehybridised with a full-length 741-bp rat *Aph-1aS* cDNA probe detecting both the short and long isoform of *Aph-1a* (*Aph-1aS* of ~2.2 kb and *Aph-1aL* of ~1.8

kb, respectively). For quantification, hybridisation signals were analysed using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom).

Quantitative RT-PCR

For quantitative RT-PCR, first-strand cDNA was prepared from 2 µg of DNase I treated total RNA (isolated as described above) using Superscript II reverse transcriptase (Invitrogen). PCR samples contained 1X SYBR Green buffer, 3 mM $MgCl_2$, 0.4 mM dUTP and 0.2 mM each of dATP, dCTP and dGTP, 0.6 U AmpliTaq Gold (all from Applied Biosystems), 0.6 µM each oligonucleotide primer (Biolegio) and 1/20 synthesised cDNA in a 25-µl volume. Quantitative PCR was performed in a PE GeneAmp 5700 apparatus with conditions as follows: 10 min at 94 °C, then 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. *ß-Actin* was amplified from all samples to normalize expression. A control (no template) was included for each primer set. Data sets were analysed with Sequence Detection System 1.3 software. The following primers were used: *Aph-1b* (448-671): 5'-GTGATTCTCCTCAGTTCTTCCTTAATTC and 5'-GCCCATGAGCACCATGATTATAT; *Aph-1a* (547-670): 5'-AGAGGAGACGG-TACTGGGCTTT and 5'-ATGGAAACGGTGACTGCATAGA; *ß-actin* (346-435): 5'-CGTGAAAAGATGACCCAGATCA and 5'-AGAGGCATACAGGGACAAC-ACA; numbers between brackets are nucleotide positions from start ATG). The *Aph-1b* primers detected all transcripts from the *Aph-1b* genes and the *Aph-1a* primers detected both *Aph-1aS* and *-1aL* mRNA. All PCR products were generated over intron-exon boundaries.

Western blot analysis

Protein extractions and immunoblottings were performed as previously described (Herreman et al., 2003). Briefly, selected brain regions and tissues were dissected from pups at PND 13 and homogenised in 50 mM Tris (pH8.0), 150 mM NaCl and 0.5% NP-40 and postnuclear fractions were isolated by centrifugation at 10,000 x *g* for one hour at 4°C. Proteins in the supernatant were quantified using a standard Bradford assay (Pierce) and 20 µg protein was loaded per lane on Tris-Tricine SDS-PAGE gels (in case of APP, APLP1 and APLP2) or on Tris-Glycine SDS-PAGE gels (for NRG2, ErbB4, p75 and tubulin) and transferred to nitrocellulose or PVDF membranes for Western blot detection. This Western blotting procedure allowed the detection of the C-terminal fragments (CTFs) of APP (using the C-terminally directed antibody C87, 1:3000) (Coolen et al., 2005), APLP1 (antibody CT-11, 1:2000) (Thinakaran et al., 1995) , APLP2 (antibody AP-tail, 1:2000) (Sester et al., 2000) and NRG2 (anti-proNRG, 1:2000) (Montero et al., 2002), the intracellular domain (ICD) of ErbB4 (antibody C-18, 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) and both the CTF and ICD of p75 (anti-p75-ICD, 1:3000) (Mahadeo et al.,

1994). The antibody against β-tubulin (antibody E7, 1:3000) (Chu and Klymkowsky, 1989) was used as an internal loading control. All antibodies were tested on tissue samples from five I/I rats of different nests and five III/III rats of different nests. Identical tissue lysates were used to test the different γ-secretase substrate levels. For quantification, hybridisation signals were analysed using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom).

Statistics

Data obtained by quantitative RT-PCR were analysed per tissue by means of an independent samples T-test. Data from the Western blot analyses were analysed per substrate by means of a univariate analysis of variance (ANOVA) with the dependent variable density (values normalised against tubulin) and the fixed factors genotype (I/I and III/III) and tissue (e.g. olfactory bulb, spinal cord, or cortex). Where appropriate, data were further analysed per tissue by means of a one-way ANOVA on genotype. A probability of $P < 0.05$ was considered statistically significant. All statistical analyses were performed with the SPSS 12.0.1 software program (SPSS Inc., Chicago, Illinois).

Results

Aph-1a and Aph-1b mRNA levels in I/I and III/III rat tissues

Northern blot analysis revealed high levels of *Aph-1b* mRNA in most III/III rat tissues analysed, whereas in the same tissues of I/I rats the levels were not or hardly above background (Figure 1A). In contrast, the mRNA levels of *Aph-1aS* and *-1aL* did not differ between I/I and III/III rat tissues, also not in the tissues with large differences in *Aph-1b* expression. Furthermore, the ratios between the *Aph-1b* and *Aph-1a* mRNA levels greatly varied among the III/III rat tissues. Tissues such as the olfactory bulb, pons/medulla, testis and lung showed high *Aph-1b/-1a* ratios, while the cortex, hippocampus, spinal cord, stomach, large intestine and thymus had moderate ratios, and the cerebellum, striatum, (hypo)thalamus, eye, heart, muscle, small intestine, spleen, liver and pancreas showed low ratios (Figure 1A). Real-time quantitative RT-PCR analysis of *Aph-1b* mRNA expression in cerebellum, olfactory bulb, cortex and lung revealed significant reductions in these tissues of I/I compared to III/III rats ($P < 0.05$), with the largest difference observed in the olfactory bulb (~8-fold reduction) (Figure 1B). The four tissues did not differ in *Aph-1a* mRNA levels between the two rat lines; the cerebellum showed the highest *Aph-1a* mRNA expression levels (one-way ANOVA followed by a post-hoc Bonferroni test: $P < 0.05$). The quantitative RT-PCR data confirmed the *Aph-1b/-1a* ratios obtained through Northern blot analysis (olfactory bulb and lung displayed high ratios, cortex showed moderate ratios, and cerebellum had low ratios) (Figure 1B).

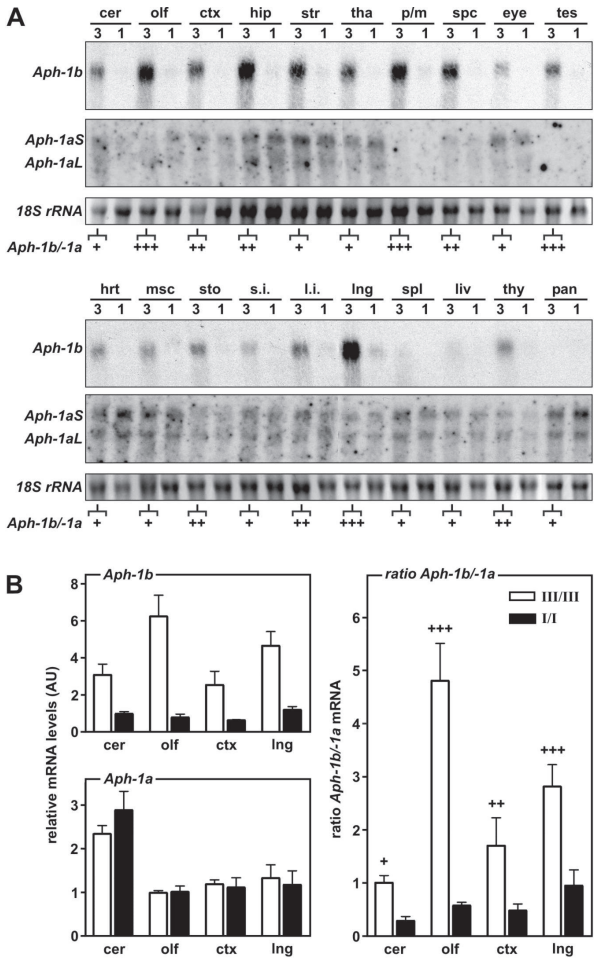
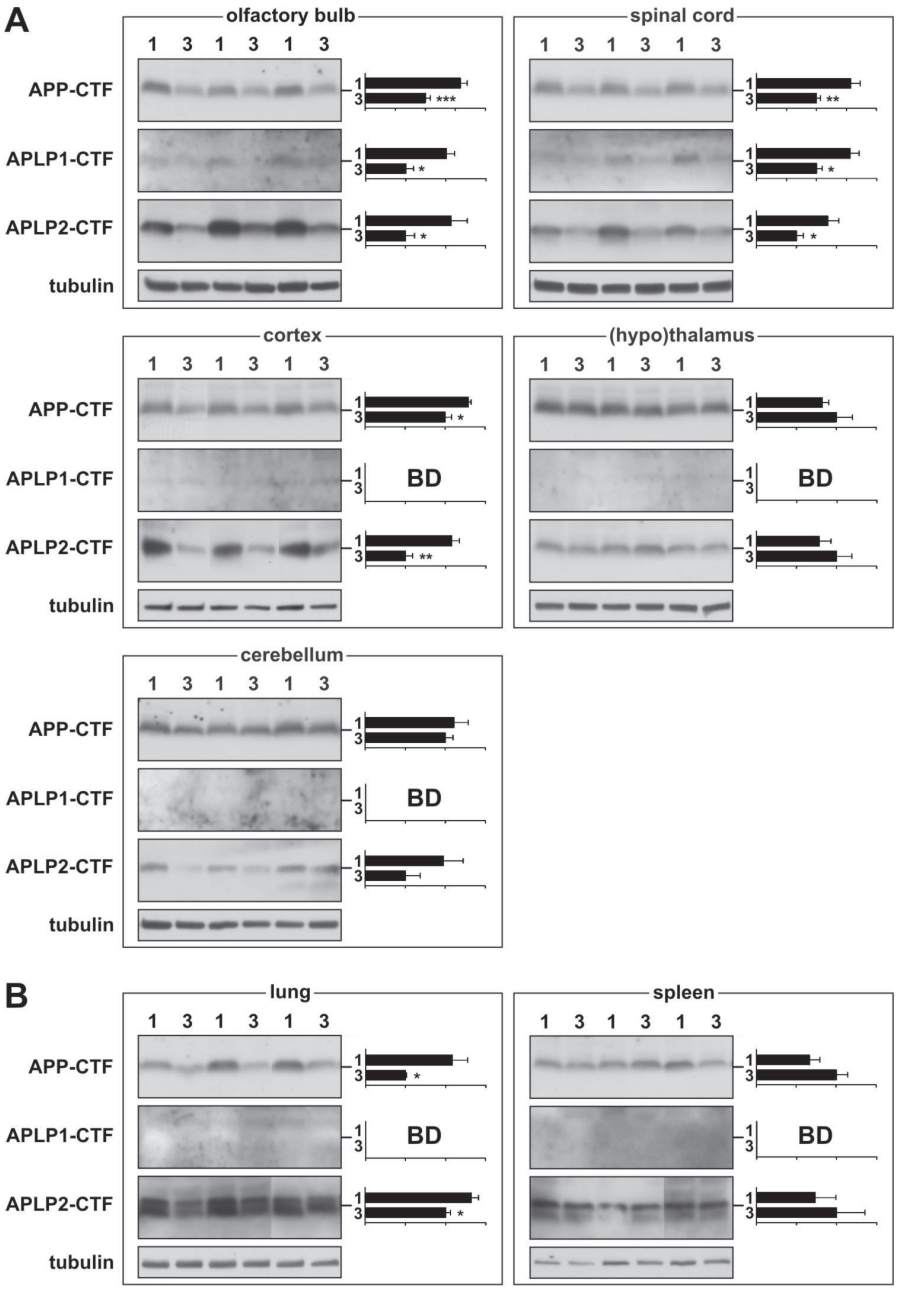


Figure 1. *Analysis of Aph-1a and -1b mRNA expression in various tissues of I/I and III/III rats. (A) Northern blot analysis of total RNA from the respective tissues of I/I and III/III rats. The blot was hybridised with a full-length rat Aph-1b cDNA probe and subsequently, following stripping, rehybridised with a full-length rat Aph-1a cDNA probe; the Aph-1a probe detected both the short and long isoform (Aph-1aS and -1aL, respectively). The 18S rRNA signal was used as a control for RNA loading and integrity. The Aph-1b/-1a intensity ratio was determined for every III/III rat tissue and categorised as high (Aph-1b/-1a > 2.0; +++), moderate (1.0 < Aph-1b/-1a ≤ 2.0; ++) or low (Aph-1b/-1a ≤ 1.0; +). Tissues used were cerebellum (cer), olfactory bulb (olf), cortex (ctx), hippocampus (hip), striatum (str), (hypo)thalamus (tha), pons/medulla (p/m), spinal cord (spc), eye, testis (tes), heart, (hrt), muscle (msc), stomach (sto), small intestine (s.i.), large intestine (l.i.), lung (lng), spleen (spl), liver (liv), thymus (thy) and pancreas (pan) of PND 9 III/III (3) or I/I (1) rats. (B) Real-time quantitative RT-PCR on total RNA from a number of I/I and III/III rat tissues. Tissues examined were cerebellum (cer), olfactory bulb (olf), cortex (ctx) and lung (lng). The Aph-1b mRNA levels of I/I compared to III/III rats were significantly reduced in the four tissues analysed (P < 0.05), whereas Aph-1a mRNA levels did not differ between the rat lines. The Aph-1b/-1a ratios calculated from the quantitative RT-PCR data were categorised as high (Aph-1b/-1a > 2.0; +++), moderate (1.0 < Aph-1b/-1a ≤ 2.0; ++) or low (Aph-1b/-1a ≤ 1.0; +). The Aph-1a primer set detected both Aph-1aS and -1aL mRNA. Results (n = 3; plus s.e.m.) were normalised towards β-actin and are expressed as arbitrary units (AU).*

γ-Secretase cleavage activity towards the APP superfamily members in I/I and III/III rat tissues

Since the Aph-1 protein is an essential component of the γ-secretase complex, we were interested in the effect of the differential mRNA expression of *Aph-1b* on the proteolytic cleavage activity of the complex in I/I and III/III rat tissues. To this end, the levels of cleavage products of various γ-secretase substrates were examined by Western blot analysis using antibodies directed against the C-terminal regions of the substrates. In general, the proteolytic processing of a γ-secretase substrate starts with shedding of its extracellular domain, leaving a C-terminal fragment (CTF) that is subsequently cleaved by γ-secretase to its ICD. One of the best-known substrates of γ-secretase is the Alzheimer's disease-linked APP protein. APP is part of the APP superfamily that in mammals includes the two APP-like proteins APLP1 and APLP2. We compared the γ-secretase cleavage activities towards the APP superfamily members in tissues of III/III rats having different *Aph-1b/-1a* ratios (high: olfactory bulb and lung; moderate: spinal cord and cortex; low: [hypo]thalamus, cerebellum and spleen) with the activities in the corresponding I/I rat tissues. Statistical analysis of the levels of direct γ-secretase substrates (CTFs) using a univariate analysis of variance (ANOVA) revealed a genotype effect for all three APP superfamily members (APP-CTF: $F(1,62) = 15.9$ $P < 0.05$; APLP1-CTF: $F(1,13) = 22.4$ $P < 0.05$; APLP2-CTF: $F(1,46) = 17.6$ $P < 0.05$). Subsequent one-way ANOVA analysis showed that the CTF levels were significantly increased in the olfactory bulb (APP: 1.6-fold [$P < 0.01$]; APLP1: 2.0-fold [$P < 0.05$]; APLP2: 2.1-fold [$P < 0.05$]), the lung (APP: 2.2-fold [$P < 0.05$]; APLP2: 1.3-fold [$P < 0.05$]), the spinal cord (APP: 1.6-fold [$P < 0.02$]; APLP1: 1.6-fold [$P < 0.05$]; APLP2: 1.8-fold [$P < 0.05$]) and the cortex (APP: 1.3-fold [$P < 0.05$]; APLP2: 2.2-fold [$P < 0.02$]) of I/I compared to III/III rats. No significant differences in the CTF levels were observed in the (hypo)thalamus, cerebellum and spleen of I/I and III/III rats, that is in tissues with a low *Aph-1b/-1a* ratio (Figure 2).

Figure 2. *Western blot analysis of the γ-secretase cleavage products derived from the APP, APLP1 and APLP2 proteins in various tissues of I/I and III/III rats. (A) The levels of the C-terminal fragments (CTFs) of amyloid-β precursor protein (APP), and the APP-like proteins APLP1 and APLP2 (sizes of all three CTFs ~10 kDa) were analysed in neuronal tissues of PND 13 I/I (1) and III/III (3) rats using specific antibodies. Tissues used were the olfactory bulb (high Aph-1b/-1a ratio), spinal cord and cortex (moderate ratio), and (hypo)thalamus and cerebellum (low ratio). (B) Levels of APP-, APLP1- and APLP2-CTF were analysed in the lung (high Aph-1b/-1a ratio) and spleen (low ratio) of PND 13 I/I and III/III rats. In each case, tubulin (~55-kDa) was used for normalisation. Bars represent quantifications in arbitrary units of normalised CTF signals of five tissue samples with the average level in III/III rat tissues set to 1. The levels of the APP, APLP1 and APLP2 holoproteins were similar in the I/I and III/III rat tissues. Significant differences in CTF levels between the I/I and III/III rats for the three APP superfamily members were found in the olfactory bulb and spinal cord, and for APP and APLP2 in the cortex, (hypo)thalamus and lung. *:$P < 0.05$; **:$P < 0.02$; ***: $P < 0.01$; $n = 5$, with the five rats per genotype from different nests; plus s.e.m.; BD: below detection.*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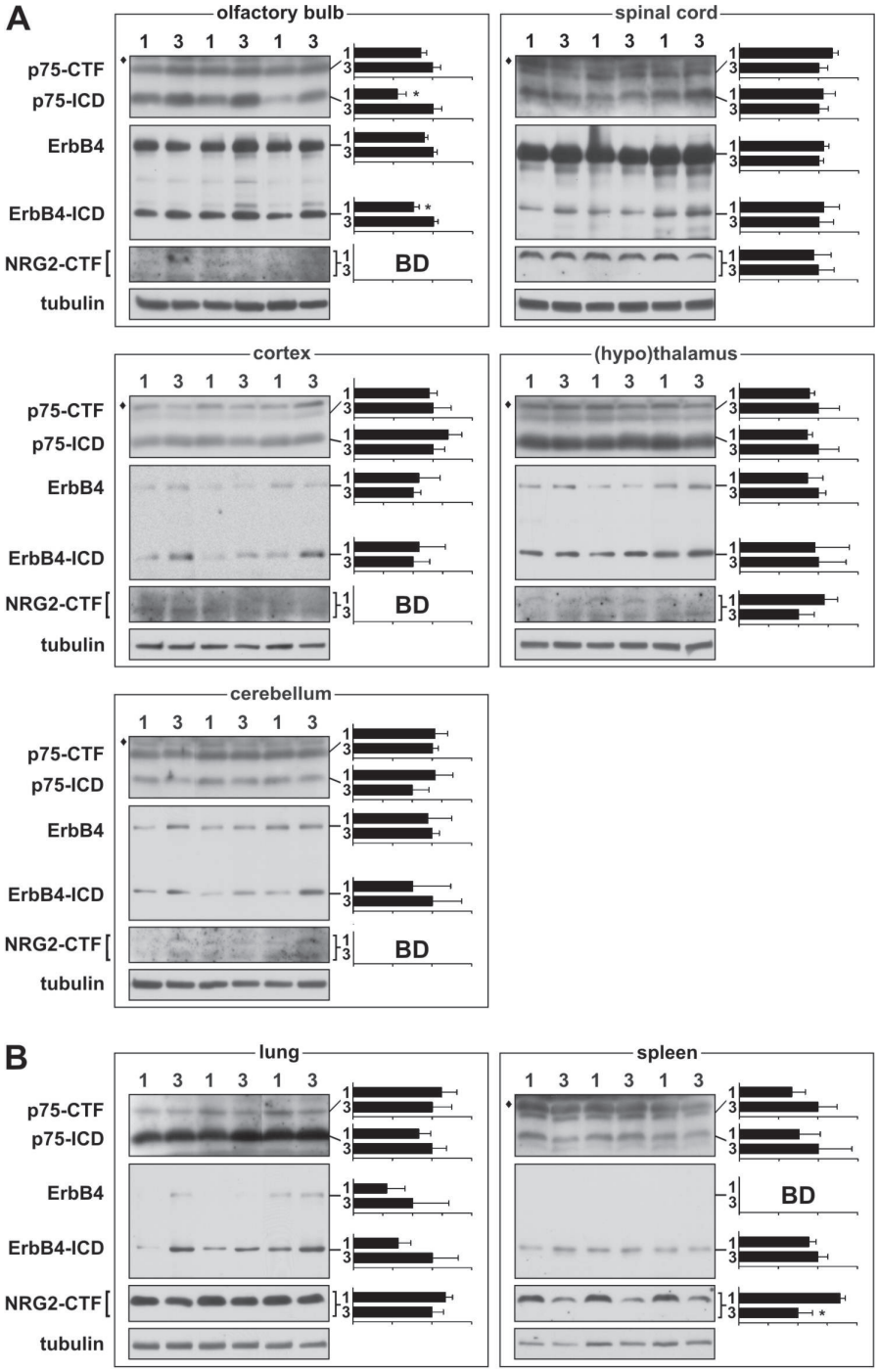
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γ-Secretase cleavage activity towards p75, ErbB4 and NRG2 in I/I and III/III rat tissues

To examine whether substrates other than the APP superfamily members showed affected levels of their γ-secretase cleavage products in I/I compared to III/III rats, we next analysed the cleavages of p75, ErbB4 and NRG2. Although no significant differences were found in the univariate ANOVA for the cleavage products of p75, ErbB4, or NRG2, visual inspection of the data prompted us to perform a one-way ANOVA per tissue. The levels of p75-CTF were similar in all I/I and III/III rat tissues tested, whereas p75-ICD showed significantly reduced levels only in the olfactory bulb of the I/I rats (1.8-fold [$P < 0.05$]). The olfactory bulb was also the only tissue with significantly reduced ErbB4-ICD levels when comparing I/I and III/III rat tissues (1.3-fold [$P < 0.05$]). The CTF levels of NRG2 were similar in all tissues examined (Figure 3).

Discussion

Over- and under-expression studies with transfected cells as well as analyses of knockout mice have shown that the γ-secretase complex requires at least four protein components to display cleavage activity, namely presenilin (PS-1 or -2), nicastrin, PEN-2 and Aph-1 (Aph-1aS, -1aL or -1b) (De Strooper et al., 1998; Yu et al., 2000; Francis et al., 2002; Edbauer et al., 2003; Kim et al., 2003; Lai et al., 2003; Serneels et al., 2005). In the present study, we examined the mRNA expression levels of *Aph-1aS*, *-1aL* and *Aph-1b*, and the effects of the differential *Aph-1b* expression on

Figure 3. *Western blot analysis of the γ-secretase cleavage products derived from p75, ErbB4 and NRG2 in various tissues of I/I and III/III rats. (A) The levels of the C-terminal fragments (CTFs) of p75 neurotrophin receptor (~30-kDa, p75-CTF) and neuregulin-2 (~20/25-kDa, NRG2-CTF) were analysed in neuronal tissues of PND 13 I/I (1) and III/III (3) rats. For p75 and neuregulin receptor ErbB4, levels of a γ-secretase cleavage end product, namely the intracellular domains (~25-kDa, p75-ICD and ~80-kDa, ErbB4-ICD, respectively), are included. Tissues used were the olfactory bulb (high Aph-1b/-1a ratio), spinal cord and cortex (moderate ratio), and (hypo)thalamus and cerebellum (low ratio). (B) Levels of p75-CTF, p75-ICD, ErbB4-ICD and NRG2-CTF were analysed in lung (high Aph-1b/-1a ratio) and spleen (low ratio) of PND 13 I/I and III/III rats. Tubulin (~ 55-kDa) was used for normalisation. Bars represent quantifications in arbitrary units of normalised signals of five tissue samples with the average level in III/III rat tissues set to 1. While the levels of the holoproteins of p75 and ErbB4 were similar in all I/I and III/III rat tissues tested, the levels of intact NRG2 were significantly increased in the (hypo)thalamus and spleen of the I/I rats (2.7-fold [$P < 0.01$], and 2.3-fold [$P < 0.05$], respectively. The levels of p75-ICD and ErbB4-ICD were significantly different only in the olfactory bulb of I/I and III/III rats (1.8-fold [$P < 0.05$], and 1.3-fold [$P < 0.05$], respectively). Although the NRG2-CTF levels were different in the spleen of the I/I and III/III rats, dividing the CTF levels by the NRG2 holoprotein levels showed no affected γ-secretase cleavage activity. *: $P < 0.05$; **: $P < 0.02$; ***: $P < 0.01$; $n = 5$, whereby the five rats per genotype were taken from different nests; plus s.e.m.; ♦: non specific product; BD: below detection.*

the cleavage activity of the γ-secretase complex in rats with one or three *Aph-1b* gene copies (I/I and III/III rats, respectively). Since a clear reduction of the *Aph-1b* mRNA levels in I/I compared to III/III rats was found, while the *Aph-1aS* and *-1aL* mRNA levels were similar between the two rat lines, the *Aph-1a* paralogues do not compensate for the reduced expression of *Aph-1b*. The ratios between the *Aph-1b* and *-1a* mRNA levels greatly varied among the III/III rat tissues. Tissues that normally have a high *Aph-1b/-1a* ratio displayed clear differences in γ-secretase cleavage activity when comparing I/I and III/III rats, whereas the activity in tissues with a low ratio was not or hardly affected. Furthermore, within a particular tissue the processing of the various substrates tested was not affected to the same extent. This suggests that *in vivo* Aph-1a and -1b are not functionally redundant but are each involved in the preferential cleavage of a subset of γ-secretase substrates (Figure 4).

We have previously found that the *Aph-1b* rat genotypes segregated with a number of behavioural phenotypes (Coolen et al., 2005), and that rats with the natural *Aph-1b* knockdown (APO-SUS) display alterations in brain information processing (prepulse inhibition and latent inhibition), locomotor activity in response to novelty, fleeing and problem-solving behaviour, and hypothalamus-pituitary-adrenal axis response to stress (Cools et al., 1990; Coenders et al., 1992; Ellenbroek et al., 1995; Rots et al., 1995). It would have been of great interest to compare this complex behavioural phenotype with the phenotype of mice generated by partial gene inactivation and thus reduced expression of the other γ-secretase components (heterozygous knockout mice), but behavioural studies on such models have not yet been performed. Unfortunately, complete knockouts of *PS1*, *nicastrin* and *Aph-1a* are lethal (Shen et al., 1997; Li et al., 2003; Ma et al., 2005; Serneels et al., 2005), and although *PS2* as well as *Aph-1b* null mutations are viable and healthy, they have not been analysed for the features observed in APO-SUS rats (Donoviel et al., 1999; Herreman et al., 1999; Serneels et al., 2005) and a *PEN-2* knockout mouse has not yet been generated. However, the behavioural phenotypes of a number of mice with altered expression of a substrate of γ-secretase have been examined and some of these show an interesting overlap with the APO-SUS phenotype. For example, heterozygous *ErbB4* or *NRG1* knockout mice (with an overall ~50% reduction of mRNA expression) are also hyperactive in an open field test and show an impaired prepulse inhibition (Gerlai et al., 2000; Stefansson et al., 2002), whereas reduced expression of Notch had no effect on the open field behaviour (Costa et al., 2003). Conversely, overexpression of human APP751 or APP-CTF caused a general hypoactivity in mice (D'Hooge et al., 1996; Lalonde et al., 2002). Behavioural studies on *APLP1*, *APLP2*, *p75* or *NRG2* heterozygous knockout or transgenic mice have not yet been described.

In our studies, we further found that of the brain tissues examined, the olfactory

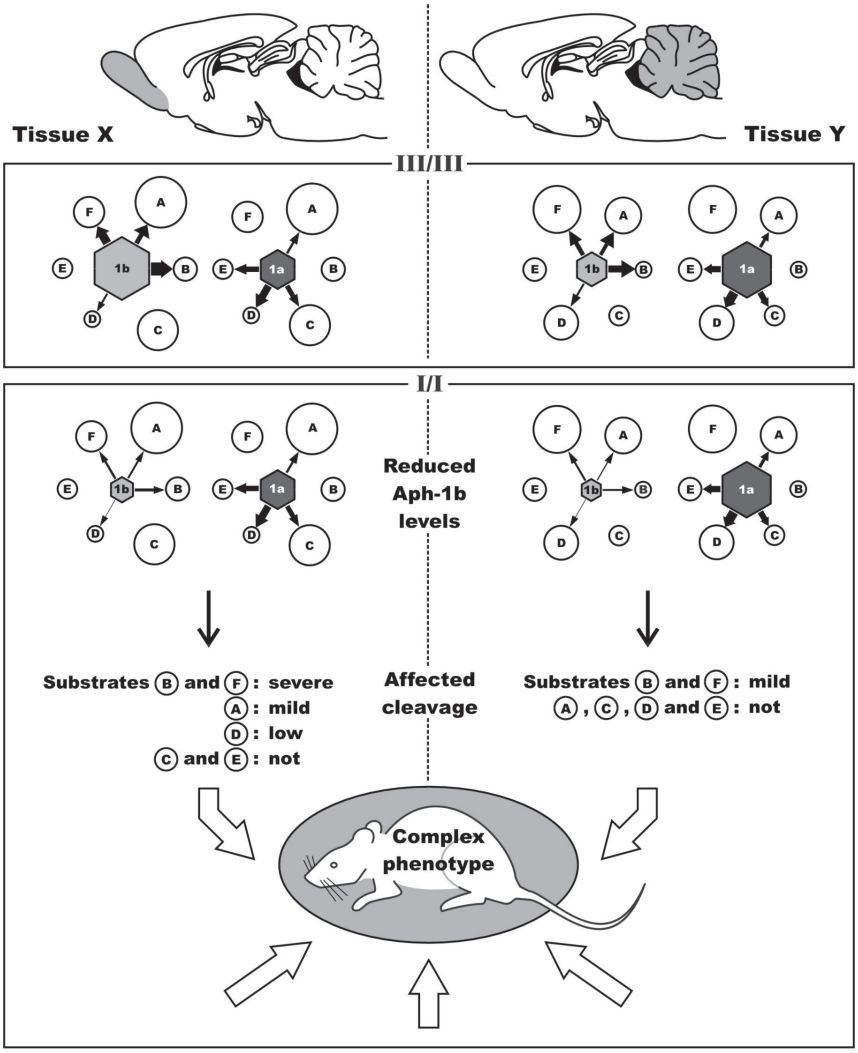

Figure 4. *Schematic representation of the tissue- and substrate-specific changes in γ-secretase activity upon reduced expression of Aph-1b, affecting a great variety of signalling pathways and resulting in a complex phenotype. Reduction of Aph-1b levels (in I/I compared to III/III rats) causes changes in γ-secretase complex compositions; complexes containing Aph-1b (termed 1b) become less available (smaller hexagonal sizes), whereas Aph-1a containing complexes remain unaffected (1a; same hexagonal sizes). These changes affect γ-secretase cleavage activity (indicated by changes in arrow thickness), but only towards a limited number of substrates and only in certain tissues. The sizes of the circles reflect the different substrate levels in the various tissues and the thickness of the arrows corresponds to the activity towards a specific substrate. For clarity, only two types of γ-secretase complexes (1a and 1b) and six substrates (A through F) are shown; in mammals, six γ-secretase complexes with different subunit compositions can be formed and over 15 substrates are known thus far. Since the various substrates are part of diverse (neuro-)developmental signalling pathways, the reduced expression of a single gene (Aph-1b) eventually results in a complex phenotype, which is generally thought to have a multigenic origin.*

bulb of the I/I rats displayed the most severely affected γ-secretase cleavage activity. This tissue showed significantly changed levels of the γ-secretase cleavage products of the three APP superfamily members, as well as of ErbB4 and p75. The olfactory bulb not only contains relatively high expression levels of PS1 and PS2, and of the γ-secretase substrates APP, APLP2 and p75 (Thinakaran et al., 1995; Lee et al., 1996; Page et al., 1996; Tisay et al., 2000), but also shows high binding affinity for γ-secretase ligands (Yan et al., 2004). Removal of the olfactory bulb from normal rats has been accepted as a model for agitated depression (reviewed in Kelly et al., 1997; Harkin et al., 2003). These so-called OBX rats show a number of behavioural changes, such as hyperactivity in an open field test, increased open arm entries in the elevated plus-maze, impairment in passive-avoidance learning as well as impaired acquisition in aversive learning, which can be reversed by chronic treatment with antidepressant drugs. Furthermore, OBX rats display alterations in the functioning of the HPA-axis, the immune system, thymus and spleen weight, and self-administration of drugs. Intriguingly, all of these phenotypic features are also observed in APO-SUS rats (Ellenbroek and Cools, 2002; Coolen et al., 2005). Of further interest is that human neurological disorders, such as Alzheimer's disease and schizophrenia, are characterised by olfactory dysfunction (reviewed in Hawkes, 2003; Moberg and Turetsky, 2003). Still, at present it is not clear to what extent the differences in γ-secretase cleavage activity found in tissues other than the olfactory bulb, such as the cortex, spinal cord and lung, have also contributed to the complex phenotype of the APO-SUS rats.

In conclusion, the differential expression of *Aph-1b* in the I/I and III/III rats caused substrate-specific alterations in γ-secretase cleavage activity, particularly in tissues with relatively high *Aph-1b* levels. We conclude from our studies on a natural *Aph-1b* knock-down in the rat that a subtle imbalance in the expression of a γ-secretase component gives rise to subtle changes in the proteolytic processing of a number of γ-secretase substrates that occur in multiple tissues (Figure 4). Thus, a single gene defect may affect a great variety of (neuro)developmental signalling pathways, resulting in a complex phenotype that is generally thought to have a multigenic origin. Furthermore, the γ-secretase complex, generally known because it is linked to Alzheimer's disease (a neurodegenerative and ageing disorder), may also be associated with (neuro)developmental disorders that become apparent much earlier in life.

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Chapter A4

Identification of genetic and epigenetic variations in a rat model for neurodevelopmental disorders

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Abstract

A combination of genetic variations, epimutations and environmental factors may be involved in the etiology of complex neurodevelopmental disorders like schizophrenia. To study such disorders, we use apomorphine-unsusceptible (APO-UNSUS) Wistar rats and their phenotypic counterpart apomorphine-susceptible (APO-SUS) rats that display a complex phenotype remarkably similar to that of schizophrenic patients. As the molecular basis of the APO-SUS/UNSUS rat model, we recently identified a genomic rearrangement of the *Aph-1b* gene. Here, we discovered between the two rat lines differences other than the *Aph-1b* gene defect, including a remarkable cluster of genetic variations, two variants corresponding to topoisomerase II-based recombination hot spots and an epigenetic (DNA methylation) difference in cerebellum and (hypo)thalamic but not hippocampal genomic DNA. Furthermore, genetic variations were found to correlate with the degree of apomorphine susceptibility in unselected Wistar rats. Together, the results show that a number of genetic and epigenetic differences exist between the APO-SUS and -UNSUS rat genomes, raising the possibility that in addition to the *Aph-1b* gene defect the newly identified variations may also contribute to the complex APO-SUS phenotype.

Introduction

Schizophrenia is a neurodevelopmental disorder affecting nearly 1% of the world's population (Jablensky et al., 1987), and is characterized by positive and negative symptoms (Kay and Opler, 1987). The aetiology of schizophrenia and other related disorders, such as schizoaffective and bipolar disorder, is still unclear. Twin, family and adoption studies have suggested that complex interactions at the genetic and environmental level underlie the aetiology of schizophrenia (Gottesman, 1991). It is thought that gene variations by themselves do not result in schizophrenia, but they can establish a predisposition status that, when combined with environmental stressors, may lead to schizophrenia pathogenesis. Numerous environmental factors, such as viral infections (Mednick et al., 1988), insufficient folate and methionine levels (Regland, 2005), or repeated psychological stress (Goldstein, 1987), can influence brain development of prenatal or early postnatal individuals with a genetic predisposition for neuropsychiatric disorders. Due to the heterogeneity in genetic and environmental interactions, most of the genes and pathways for schizophrenia and for other complex disorders are still unknown.

To get insight into the gene (or genes) that may be involved in schizophrenia pathogenesis, a rat model was developed with schizophrenia-like features. This model was based on the behavioural response of Wistar rats to the dopamine agonist apomorphine (Cools et al., 1990). The apomorphine-susceptible (APO-SUS) rat line displayed many features of psychopathology, with similar disturbances at the behavioural, physiological, endocrinological and pharmacological level as seen in schizophrenics (Ellenbroek and Cools, 2002). For example, APO-SUS rats have a reduced prepulse inhibition and latent inhibition (Ellenbroek et al., 1995), display a higher plasma release of adrenocorticotropin (ACTH) and corticosteroids in response to novelty (Rots et al., 1995), are more sensitive to dopamimetic drugs (Ellenbroek et al., 2000), and have a higher susceptibility to inflammatory and infectious diseases when compared to apomorphine-unsusceptible (APO-UNSUS) rats (Kavelaars et al., 1997). We therefore wondered about the molecular-genetic basis underlying the APO-SUS/-UNSUS rat model and recently identified a genetic difference between the two rat lines (Coolen et al., 2005). Whereas APO-UNSUS rats harbour three gene copies of the γ-secretase component *Aph-1b*, APO-SUS rats have only one or two copies. This gene-dosage imbalance was due to an unequal crossing over event (nonallelic homologous recombination) between two direct repeats (a segmental duplication) within the *Aph-1b* locus. In addition, we observed a direct link between the *Aph-1b* genotypes and a number of phenotypic APO-SUS and –UNSUS characteristics (Coolen et al., 2005). Approximately 10 years after developing the APO-SUS and –UNSUS lines a second, independent breeding procedure was started that resulted in

rats with features similar to those displayed by the original APO-SUS and -UNSUS rat lines (Ellenbroek and Cools, 2002). Interestingly, the replicated rat lines also resulted in APO-UNSUS rats with three *Aph-1b* gene copy numbers and APO-SUS rats with only one or two gene copies (Coolen et al., 2005).
In the present study, we wondered whether genetic variations other than the *Aph-1b* gene-dosage imbalance may be present between the APO-SUS and -UNSUS rats, and whether epigenetic factors may be involved as well. Epigenetics has been defined as heritable changes in gene expression that do not occur by changes in the DNA sequence, but by modifications in DNA methylation and chromatin remodeling (Wolffe and Matzke, 1999), or, in its widest sense, as any change in an organism that is not due to genetic factors (Van de Vijver et al., 2002). Increasing evidence suggests that epigenetic modifications play a role in disease susceptibility (reviewed by Jirtle and Skinner, 2007). We used the arbitrarily primed-polymerase chain reaction (AP-PCR) fingerprinting technique (Welsh and McClelland, 1990) to analyse the genomes and epigenomes (DNA methylation) of the APO-SUS and –UNSUS rats. Comparison of the AP-PCR fingerprints generated from the genomic DNAs of the two rat lines revealed genetic as well as epigenetic alterations and we conclude that, besides in the *Aph-1b* locus, a number of other variations are present in the APO-SUS and –UNSUS genomes and epigenomes.

Materials and Methods

Experimental animals

The generation of the APO-SUS and -UNSUS rat lines with a high or low susceptibility for apomorphine, respectively, has been described previously (Cools et al., 1990). The present experiments were performed with male APO-SUS and –UNSUS rats belonging to the 32nd (original lines) and 18th (replicate lines) generation. At post-natal day 60 (PND60), APO-SUS and -UNSUS rats were sacrificed and the hippocampus, cerebellum and the combined thalamus/hypothalamus (further denoted as (hypo) thalamus) were isolated. To establish their apomorphine susceptibility, unselected male Wistar rats of the Nijmegen outbred population (PND60) were injected with apomorphine (1.5 mg/kg s.c.) and gnawing scores were measured in a gnawing box for 45 minutes, as described previously (Cools et al., 1990). Immediately following the measurements, the rats were sacrificed and the same tissues (hippocampus, cerebellum and (hypo)thalamus) were removed. All rats were bred and reared in the Central Animal Facility of the Radboud University Nijmegen under approved animal protocols and in accordance with institutional guidelines.

Arbitrarily primed-PCR

Genomic DNAs were isolated from hippocampus, cerebellum and (hypo)thalamus using standard procedures involving the use of proteinase K and phenol extraction. Two micrograms of genomic DNA were digested with 20 units of *Rsa*I, 20 units *Rsa*I in combination with the methylation-sensitive enzyme *Hpa*II, or 20 units *Rsa*I and *Msp*I (MBI Fermentas) in a total volume of 40 µl at 37°C for 16h. *Hpa*II does not cut DNA if the internal cytosine (C<u>C</u>GG) is methylated, whereas *Msp*I is insensitive to DNA methylation. Using such combinations of methylation-sensitive and -insensitive enzymes allows genome-wide screening for differences at the genetic level (single-nucleotide polymorphisms – SNPs, duplications, insertions, deletions and recombinations) as well as the epigenetic (DNA methylation) level. Restriction enzymes were heat inactivated by incubating the reactions at 65°C for 20 min. Digested DNA (100 ng) was amplified using AP-PCR (Welsh and McClelland, 1990) with a single primer. PCRs were performed in a total volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.25% Nonidet P-40, 0.25% Tween-20, 200 µM each of the four deoxynucleotide triphosphates, ~1 µCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Corp.), 25 pmol of primer (AP-1: 5'-AACCCTCACCCTAACCCCGG-3', AP-7: 5'-AACCCTCACCCTAAGGCGCG-3', AP-777: 5'-CACTCCTCTAC-AAGGTGCCG-3' or Topo: 5'-GCCTCCTTGCAGGTCTTT-3'), and 0.8 units of *Taq* polymerase (MBI Fermentas). Reactions were carried out in a thermal cycler (PerkinElmer) with five cycles of low stringency (94°C for 30 sec, 40°C for 60 sec, 72°C for 1.5 min), followed by 30 cycles of high stringency (94°C for 15 sec, 55°C for 15 sec, 72°C for 1 min). Two microliters of the PCR products were analysed on high-resolution 5% polyacrylamide gels under denaturing conditions (7 M urea) for 4-4.5 h at 70 W. Gels were dried and radiolabelled DNA was visualized by autoradiography at -70°C (CEA AB, Sweden).

Cloning and sequencing of AP-PCR fragments

AP-PCR fragments generated from APO-SUS and –UNSUS rat genomic DNAs were excised from the dried gels and incubated in 50 µl MilliQ at 80°C for 10 min. The eluted DNA (two microliters) was reamplified with the same primer as used for the AP-PCR to generate sufficient amounts of template for subsequent cloning. The reactions were carried out for 40 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 1 min, under the same conditions as described in the AP-PCR protocol (except that [α-^{32}P]dCTP was not included). The PCR products were purified, cloned into the pGEM-T easy vector (Promega) and sequenced with a T7 or Sp6 primer according to the manufacturer's instructions using the ABI310 machine (Applied Biosystems).

Sequencing and genotyping of chromosomal region 9q22

A 1948-bp fragment that harbours the nucleotide sequence corresponding to product 3 was obtained by PCR on genomic DNA derived from (hypo)thalamic tissue of an APO-UNSUS rat using forward primer 5'-GGGAAGCAACGCATCCTG-3' and reverse primer 5'-CATATCAAAGCACCAAGTCCACAG-3'. The DNA was subsequently purified and directly sequenced using the ABI310 machine (Applied Biosystems). Genotyping of chromosomal region 9q22 was performed with PCR using primers specific for either the APO-SUS or APO-UNSUS genomic sequence. Briefly, PCRs were performed in a total volume of 20 µl containing 50 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.25% Nonidet P-40, 0.25% Tween-20, 200 µM each of the four deoxynucleotide triphosphates, 0.6 µM of each primer (FW: 5'- AACACTTGGACTCATTCTCACTGG-[G (SUS) or T (UNSUS)]-3 ' and RV: 5'- CCTGGATGGAATGTTGACAC-[C (SUS) or T (UNSUS)]-3'), and 0.8 units of *Taq* polymerase (MBI Fermentas). Reactions were carried out at 94°C for 60 sec, 58°C for 60 sec and 72°C for 60 sec for 35 cycles. Products were analysed on a 1% agarose gel.

Quantification and statistics

Quantification of AP-PCR products was performed using the Labworks 4.0 program (UVP BioImaging Systems, Cambridge, UK) and statistical evaluation was performed by means of an unpaired Student's *t*-test.

Results

AP-PCR DNA fingerprint patterns of the APO-SUS and APO-UNSUS rat genomes and epigenomes

In order to identify differences between the genomes and epigenomes of APO-SUS and -UNSUS rats, we performed a comparative analysis of fingerprints of AP-PCR products generated from genomic DNAs of the two rat lines. Initially, genomic DNAs isolated from APO-SUS and APO-UNSUS (hypo)thalamic tissue and digested with *Rsa*I in combination with the methylation-sensitive restriction enzyme *Hpa*II (CCGG) was analysed using arbitrary primers AP-1, AP-7 or AP-777. These primers were selected from a total set of ten primers because they gave fingerprints with reproducible and discrete products (data not shown). Typical AP-PCR fingerprints obtained with the three selected arbitrary primers are shown in figure 1. With each arbitrary primer ~30 chromosomal fragments were reproducibly amplified. DNAs digested with *Rsa*I and the methylation-insensitive enzyme *Msp*I served as controls to determine whether the observed differences were due to a differential methylation of the CCGG sequence or a genetic polymorphism in this sequence. AP-PCR analysis

with primer AP-1 revealed 16 products corresponding to fragments without an *Hpa*II site ("genetic fragments") and 23 products corresponding to fragments containing an *Hpa*II site ("epigenetic fragments"). Analysis with AP-PCR primer AP-7 showed 17 genetic and 11 epigenetic fragments, and with AP-777 primer 22 genetic and 6 epigenetic fragments.

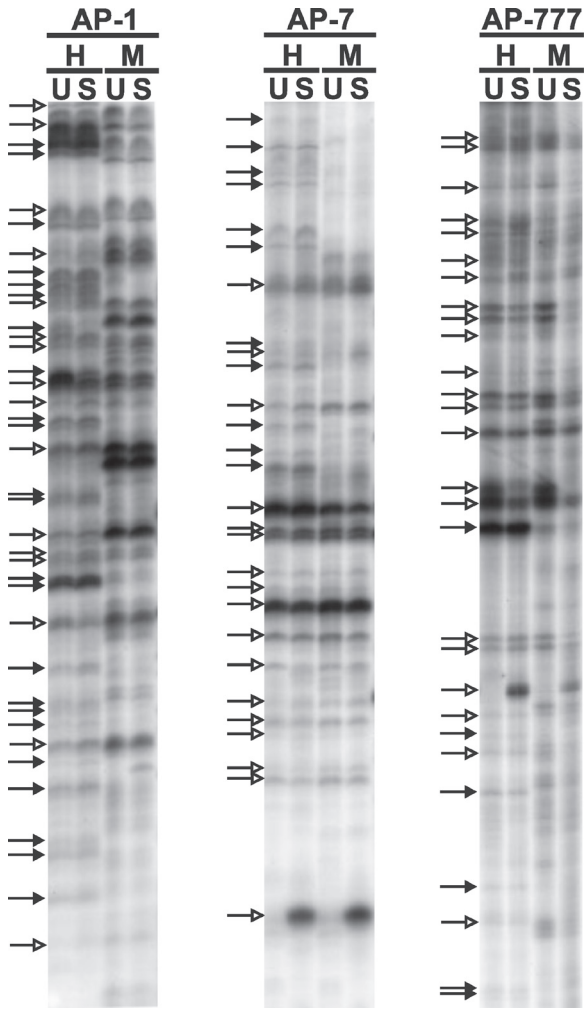

Figure 1. *AP-PCR analysis of genomic DNAs from APO-UNSUS (U) and APO-SUS (S) (hypo)thalamus. AP-PCR was performed with primers AP-1, AP-7 and AP-777 using genomic DNAs digested with RsaI and HpaII (H) or RsaI and MspI (M) as templates. Epigenetic products (methylation-sensitive and thus absent in the MspI lanes) are indicated by closed arrows and genetic products (methylation-insensitive and thus present in the MspI lanes) by open arrows.*

Genetic variations between the APO-SUS and APO-UNSUS rat genomes

Comparison of the genetic fingerprints generated with primers AP-1, -7 and -777 revealed three reproducible variations between the genomic DNAs from the original (F32) APO-SUS and –UNSUS rats, designated products 1, 2 and 3 (figure 2a). Product 1 was less prominent in the APO-SUS than in the APO-UNSUS rats, product 2 was found only in APO-SUS, while the level of product 3 was higher in the APO-SUS than -UNSUS rats. Interestingly, the three products were also present in the replicate (F18) lines and at the same levels, indicating that the replication of the APO-SUS and -UNSUS lines had resulted in a similar genotypic distribution. Next, digestions using *Rsa*I in combination with *Msp*I were used to examine whether the observed differences were due to a genetic or an epigenetic alteration. Following digestion with *Rsa*I and *Msp*I, products 1, 2 and 3 were still found, indicating that the presence of the three AP-PCR products was due to genetic differences (figure 2b).

We previously discovered that the *Aph-1b* gene-dosage imbalance between the APO-SUS and –UNSUS rats is the result of a DNA recombination event between the two *Aph-1b* genes. Furthermore, we identified the region in which the recombination occurred, namely in a region of 1106 nucleotides that is identical between the two genes and encompasses exon 5 (Coolen et al., 2005). In the present study, we decided to examine in detail the site of recombination and found a topoisomerase II binding site (5'-ACCCACCTGCTGGTGTCC-3') in the DNA region harbouring the recombination site. Topoisomerase II binding sites (with the vertebrate consensus sequence 5'-RNYNNCNNGYNGKTNYNY-3') (Spitzner and Muller, 1988) are known to be hotspots where DNA recombination events occur easily (Craig and Nash, 1983). We therefore wondered whether other topoisomerase II binding sites could have led to additional differences between the APO-SUS and –UNSUS rat genomes. Interestingly, using a primer based on the topoisomerase II binding site consensus for PCR analysis of genomic DNAs digested with *Eco*RI or *Mbo*I revealed two differences between the APO-SUS (n=3) and –UNSUS (n=2) rat genomes of the original (F32) lines, designated products 4 and 5 (figure 2c). Product 4 was present in APO-SUS but not in APO-UNSUS rat genomic DNA. In the replicate –SUS and -UNSUS lines (F18), the genomes of two APO-UNSUS rats did also not contain product 4, whereas it was present in two of the four APO-SUS rats tested. Product 5 was present in three of the four APO-UNSUS rats examined (in both the original and the replicate lines), whereas it was not observed in the seven APO-SUS rats tested (figure 2c).

Epigenetic variations between the APO-SUS and APO-UNSUS rat genomes

We then wondered whether, besides the five genetic variations, also epigenetic variations would be present between the APO-SUS and –UNSUS rat lines. AP-PCR

analysis using primer AP-1 on *Rsa*I- and *Hpa*II-digested genomic DNAs from APO-SUS and –UNSUS (hypo)thalamus revealed one epigenetic variation, designated the E1-product (figure 3a). The difference was observed in both the original APO-SUS and -UNSUS rats as well as the replicated lines. An ~1.4-fold reduced amount of the E1-product was observed in APO-SUS when compared with APO-UNSUS genomic DNAs (n=12, $p<0.05$). Since DNA methylation may be tissue specific, we decided to examine the E1-product in two other brain tissues. The level of the E1-product was ~2-fold reduced in genomic DNAs from the cerebellum of APO-SUS compared to APO-UNSUS rats (n=4, $p<0.01$), while no difference in the E1-levels was found in the hippocampus of the APO-SUS and -UNSUS rats (n=4).

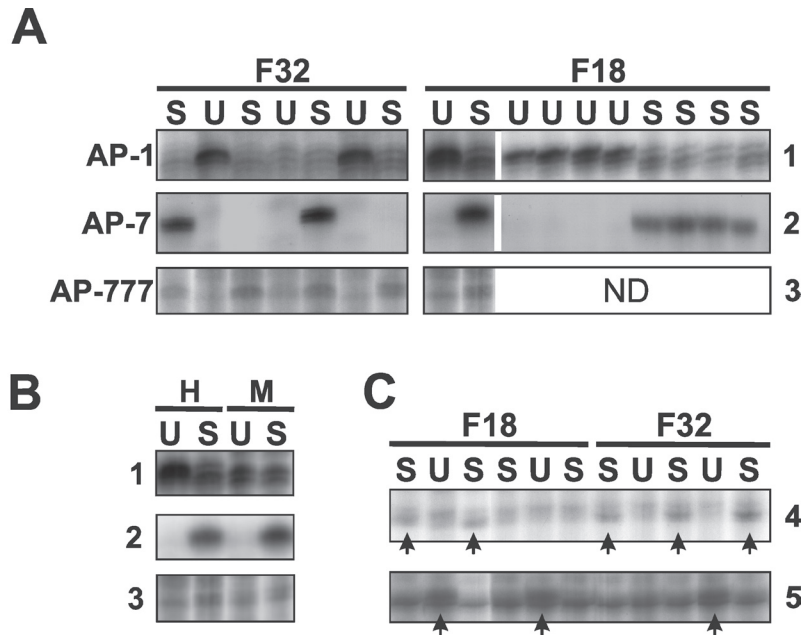

Figure 2. *Genetic variations in genomic DNAs from APO-SUS (S) and APO-UNSUS (U) (hypo)thalamus. Rats were from the original (F32) or the replicate (F18) rat lines. (A) Products generated by AP-PCR using primers AP-1 (product 1), AP-7 (product 2) or AP-777 (product 3) on genomic DNAs digested with the methylation-insensitive enzyme RsaI and the methylation-sensitive enzyme HpaII. ND = not determined. (B) Products 1, 2 and 3 generated by AP-PCR on genomic DNAs digested with RsaI and HpaII (H) or with the two methylation-insensitive enzymes RsaI and MspI (M). MspI products served as controls to determine whether products 1, 2 and 3 were due to differential methylation or to a genetic polymorphism. The fact that products 1, 2 and 3 were still present following MspI digestion indicates that they represent products without an HpaII site ("genetic fragments"). (C) Products generated by AP-PCR using a topoisomerase II binding site consensus sequence (5'-GCCTCCTTGCAGGTCTTT-3') on genomic DNAs digested with the methylation-insensitive enzymes EcoRI (product 4) or MboI (product 5). Arrows indicate increased amounts of the AP-PCR products.*

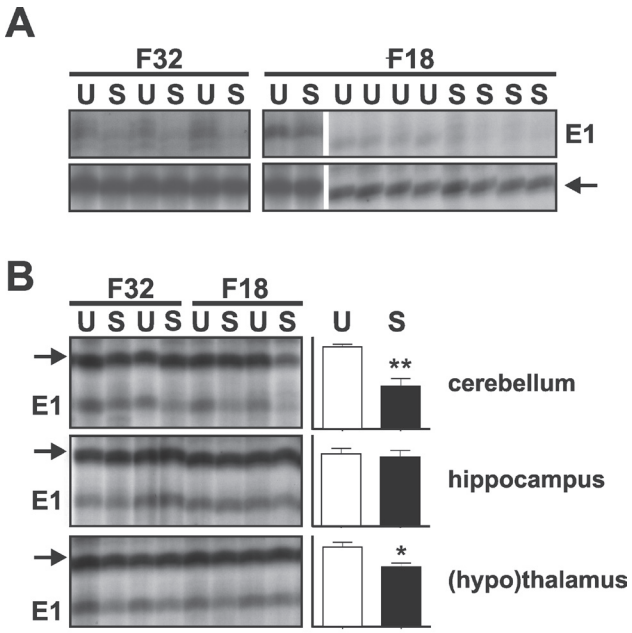

Figure 3. *Epigenetic variation in genomic DNAs from APO-SUS (S) and APO-UNSUS (U) (hypo) thalamus. Rats were from the original (F32) or the replicate (F18) rat lines. (A) Product E1 generated by AP-PCR using primer AP-1 on genomic DNAs digested with RsaI and HpaII. Arrow indicates a representative example of an AP-PCR product that did not show variable amounts using primer AP-1 (randomly chosen out of 38 products) and was used for normalization. (B) The AP-PCR E1-product generated from genomic DNAs from cerebellum, hippocampus and (hypo)thalamus. All rats were from different nests. The products indicated by the arrow were used for normalisation. Amounts for the epigenetic E1-product were significantly different between the APO-SUS and APO-UNSUS rats in the cerebellum (** $p < 0.01$; n=4, plus s.e.m.) and (hypo)thalamus (* $p < 0.05$; n=12, plus s.e.m.), but not in the hippocampus (n=4, plus s.e.m.).*

Chromosomal localizations of the genetic and epigenetic variations between the APO-SUS and –UNSUS rat genomes

To identify the locations of the genetic and epigenetic variations within the APO-SUS and -UNSUS rat genomes, AP-PCR fragments 1, 2, 3 and E1 were excised from the gel and each fragment was reamplified with the primer used for the original AP-PCR reaction. DNA sequence analysis of the amplified PCR fragments and database searches with the obtained nucleotide sequences revealed the chromosomal localisations of the four fragments; product 1 was located on chromosome 19q11, ~3.3 kb upstream of the first exon of the GAIP-interacting protein, C terminus (GIPC) gene; product 2 was part of a repeat sequence located on chromosome 2q34; product 3 was located in the first intron of the *myosin 1b* gene on chromosome 9q22; the epigenetic variation E1 was located on chromosome 6q31, downstream of the Jun dimerization protein 2 (NP_446346.1) and upstream of the ATF-like basic leucine

zipper transcriptional factor B-ATF (SF-HT-activated gene 2; XP_216745.2). Since product 3 was localized within a gene, we decided to analyse this chromosomal region in more detail. Sequence analysis of the DNA region corresponding to AP-PCR fragment 3 and its surrounding region revealed a remarkably high number of genetic variations: 10 of the 1948 base pairs analysed were different between the APO-SUS and –UNSUS genomes (figure 4). Comparison of the nucleotide sequences of the APO-SUS and –UNSUS DNA regions with the corresponding database sequence (geneID: 117057) revealed a 100% identity between the database and the APO-UNSUS sequences, indicating that the APO-SUS genome has diverged from the database sequence. Next, more APO-SUS and -UNSUS rats were genotyped for this region. All APO-UNSUS rats tested (n=5) indeed showed the database sequence, whereas the APO-SUS rats tested (n=5) all contained the relatively high number of variations in this chromosomal region.

Figure 4. *Nucleotide sequence of chromosomal region 9q22, corresponding to product 3, in APO-SUS and APO-UNSUS rats. APO-SUS/-UNSUS genomic variations are indicated between brackets; the first nucleotide represents the APO-UNSUS sequence, the second nucleotide the APO-SUS sequence. The nucleotide sequences corresponding to the annealing sites of arbitrary primer AP-777 are underlined.*

The newly identified genetic and epigenetic variations, and apomorphine susceptibility in Wistar rats

We wondered whether the molecular-genetic difference between the APO-SUS and –UNSUS rats (the *Aph-1b* gene-dosage imbalance) would also be present in the Nijmegen outbred population of Wistar rats, or if the imbalance was generated during the breeding of the rat lines. PCR analysis of the *Aph-1b* locus in the 50

Wistar rats examined revealed in all cases the presence of three copies of the *Aph-1b* gene, suggesting that the reduction of *Aph-1b* copies in the APO-SUS rats had been induced during the breeding of the rats.

We next wondered whether the newly identified genetic and epigenetic variations in the APO-SUS and -UNSUS genomes were also induced during the breeding of the two rat lines, or if these variants are already present in Wistar rats. For this purpose, we performed AP-PCR analysis of (hypo)thalamic genomic DNAs from Wistar rats using primers AP-1, -7 and –777. The fingerprints revealed the presence of the APO-SUS as well as the APO-UNSUS variants of the genetic products 1, 2 and 3 (n=8) and the epigenetic E1-product (n=4) in Wistar rats (figure 5), indicating that the newly identified variations were not induced during breeding of the APO-SUS and -UNSUS lines, but were already present in the original Wistar population.

To study whether the presence or absence of the genetic AP-PCR products observed in the APO-SUS and –UNSUS rats (products 1, 2 and 3) was linked to the apomorphine susceptibility of Wistar rats, we examined a group of five rats with low susceptibility to apomorphine (<10 gnaws per 45 min) and a highly susceptible group of three rats (>500 gnaws per 45 min). Product 1, which was less abundant in APO-SUS than -UNSUS rats, was not present in the three Wistar rats with high gnawing scores, but was also not found in two of the five Wistar rats with low gnawing scores. Of the eight rats examined, the APO-SUS-specific product 2 was found in two Wistar rats with a high and only one rat with a low degree of apomorphine susceptibility. The DNA region corresponding to product 3 was found twice as the APO-UNSUS sequence in the group consisting of the low-apomorphine-susceptible Wistar rats and once in the group of the high-apomorphine-susceptible rats (figure 5a). These results indicate that none of the three products was directly linked to the apomorphine susceptibility of the Wistar rats. However, apomorphine susceptibility may not be the result of only a single genetic variation, but rather of multiple genetic and epigenetic alterations. Interestingly, 67% of the three genetic products present in the Wistar rats with low apomorphine susceptibility were also found in the APO-UNSUS genome, and 78% of the three products in Wistar rats with high apomorphine susceptibility were APO-SUS-specific variants (figure 5a), indicating that a combination of the three genetic products may well be linked to apomorphine susceptibility. To study the link between the epigenetic E1-product and apomorphine susceptibility, two Wistar rats with low gnawing scores and two with high gnawing scores were examined for the amount of the E1-product in the cerebellum. Similar amounts of the E1-product as detected in APO-UNSUS rats were found in one Wistar rat with a low and in one Wistar rat with a high gnawing score. The amount of the E1-product detected in APO-SUS rats was found in one Wistar rat with low and in one Wistar rat with high apomorphine susceptibility, indicating that no direct link exists between the E1-product and

apomorphine susceptibility. As observed in the APO-SUS and –UNSUS rats, no variation was detected in the level of the E1-product in hippocampal genomic DNAs from the four Wistar rats tested (figure 5b).

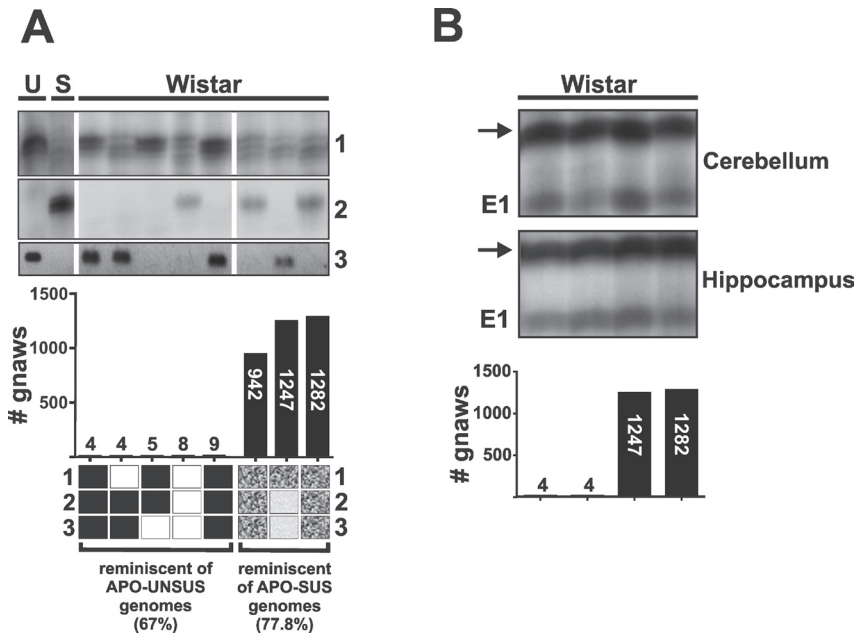

Figure 5. *AP-PCR analysis (products 1, 2 and E1) and specific PCR (product 3) analysis of genomic DNAs from Wistar rats with low or high apomorphine susceptibility. (A) The presence or absence of products 1, 2 and 3 (genetic differences) was analysed in genomic DNAs from the (hypo)thalamus of Wistar rats with low (<10 gnaws in 45 min) or high (>500 gnaws in 45 min) apomorphine susceptibility. U: APO-UNSUS, S: APO-SUS. Lower panel: the amounts of the three products present in the Wistar rats were compared with the amounts found in the APO-SUS and –UNSUS rats. ■: the genotype of the genetic products 1, 2 or 3 in the Wistar rats with low apomorphine susceptibility is similar to the products 1, 2 and 3 genotype of the APO-UNSUS rats; □: the genotype in the Wistar rats with low apomorphine susceptibility deviates from the APO-UNSUS genotype; ▩: the genotype in the Wistar rats with high apomorphine susceptibility is comparable with the genotype in the APO-SUS rat; ▨: the genotype in the Wistar rats with high apomorphine susceptibility deviates from the genotype in the APO-SUS rats. (B) AP-PCR analysis of the epigenetic E1-product (E1) on genomic DNAs from cerebellum and hippocampus of four Wistar rats. The product indicated by the arrow is an example of a product that did not show variable amounts and was used for normalisation of the E1-product. The amounts of the E1-products generated from the cerebellum or hippocampus were not different between the two Wistar rats with low gnawing scores (<10 gnaws in 45 min) and the two Wistar rats with high gnawing scores (>500 gnaws in 45 min).*

Discussion

In this study, we investigated the genetic and epigenetic background of the phenotypically well-characterized APO-SUS and APO-UNSUS rats. Unravelling the

molecular basis of this rat model may help in our understanding of complex human neurodevelopmental disorders, since many of the characteristics of the APO-SUS rat line are also observed in schizophrenic patients (Ellenbroek and Cools, 2002). We recently identified a gene-dosage imbalance in the *Aph-1b* locus of the APO-SUS and –UNSUS rats, leading to a reduced expression of the *Aph-1b* gene in APO-SUS rats and a segregation with a number of behavioural parameters (Coolen et al., 2005). Here, we used AP-PCR analysis as an approach to explore the presence of any other alterations in the two genomes and epigenomes. We indeed identified additional genetic and epigenetic variations, indicating that the *Aph-1b* locus might not be solely responsible for the observed phenotypes of the two rat lines and suggesting a multi-genetic and -epigenetic origin of the differences observed between the APO-SUS and –UNSUS rats. Importantly, we found similar AP-PCR patterns in both the original and replicate APO-SUS and –UNSUS lines. It is therefore highly unlikely that the observed variations between the APO-SUS and -UNSUS rat genomes were simply due to coincidence.

Detailed analysis of one of the newly identified genetic alterations (in the DNA region corresponding to product 3) revealed in APO-SUS rats a cluster of variations in the *myosin 1b* gene. Hence, this cluster appears to be a hotspot for genetic instability. Product 1 was located upstream of the GIPC gene encoding a protein interacting with membrane-associated and transmembrane proteins, including the dopamine receptors D2 and D3 (Jeanneteau et al., 2004). The genetic variation in this locus may therefore contribute to the differences in apomorphine susceptibility between the APO-SUS and -UNSUS rat lines. We also identified genetic alterations using a primer corresponding to a topoisomerase II binding site consensus. The rationale for this study was based on our present finding that a topoisomerase II binding site was located at the recombination site in the *Aph-1b* locus. The two newly identified, topoisomerase II-based genetic variations might point to a more general role for topoisomerase II binding sites in psychopathological mechanisms. We hypothesize that at these sites environmental factors, such as stress during early development, may cause an increase in the incidence of recombination and other mutagenic events, leading to brain dysfunction and affected behavior. Besides the genetic differences, one tissue-specific epigenetic variation was found between the APO-SUS and –UNSUS epigenomes. At present it is not clear what, if any, functional consequence should be attributed to the decrease in the methylation status of this CpG in the (hypo)thalamus and cerebellum, but not in the hippocampus, of APO-SUS relative to APO-UNSUS rats.

Earlier microarray analysis of mRNA expression in the hippocampus of the APO-SUS and -UNSUS rats (~7000 full-length sequences and ~1000 EST clusters) revealed that only *Aph-1b* was differentially expressed (Coolen et al., 2005). The

newly identified variations open the possibility that for an explanation of the background of the rat model one has to consider more genes that operate together in a multi-genetic and -epigenetic setting with several susceptibility loci. Thus, based on our present findings, more than one locus may be responsible for the complex phenotype of APO-SUS rats. Recently, it has been established that the contribution of genetic modifiers is also of importance for the outcome of a phenotype, since they can modulate the severity of the affected phenotype and the phenotypic characteristics without having a clear effect on the normal situation (Nadeau, 2001; Nadeau and Topol, 2006). Hence, as part of the genetic and epigenetic background of the APO-SUS and -UNSUS rat lines, our newly identified variants can be genetic and epigenetic modifiers influencing the phenotypic expression of the model. Insight into the genetic and epigenetic background may provide diagnostic tools, and clues for mechanisms and pathways to explain complex disorders.

Besides the multiple variations in the APO-SUS and –UNSUS rat genomes and epigenomes, *Aph-1b* will presumably be a major player in the development of the complex APO-SUS phenotype, since it has a broad cellular effect via tissue-specific cleavage of many different substrates (Coolen et al., 2006b) and is functional already during early development (Coolen et al., 2006a). Remarkably, however, the number of *Aph-1b* gene copies was not linked to apomorphine susceptibility in Wistar rats. Whereas their apomorphine susceptibility varied, all unselected Wistar rats tested harboured three copies of the *Aph-1b* gene. Therefore, genetic and epigenetic factors other than *Aph-1b* will likely contribute to the susceptibility for apomorphine. We now indeed found a correlation between the newly identified genetic variations and the apomorphine susceptibility in the Wistar population, confirming our hypothesis that apomorphine susceptibility is caused by a number of genetic and epigenetic factors. A combination of the newly identified variations may have thus initially contributed to the degree of apomorphine susceptibility in the original Wistar rat population. Subsequent apomorphine injections to determine the susceptibility for the drug during the breeding of the APO-SUS and –UNSUS lines may have acted as an environmental stressor, triggering the *Aph-1b* recombination event at the topoisomerase II binding site only in rats with a high susceptibility for apomorphine. The induced gene-dosage imbalance of the *Aph-1b* gene, probably in combination with other genetic or epigenetic factors, could then have led to the complex phenotype observed in the APO-SUS rats.

In conclusion, the present findings suggest that psychopathological disturbances may be the result of multiple genetic as well as epigenetic factors. We infer that our newly identified variations are susceptibility loci for schizophrenia-like features in the rat and may give new insights into the genetic and epigenetic background of complex neurodevelopmental disorders.

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Chapter A5

Genomic copy number variations in rats with a complex phenotype

With
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Part of this chapter has been submitted.

Abstract

Complex phenotypes are thought to have a multifactorial origin with multiple genetic variations that act in concert with environmental factors. Besides single-nucleotide polymorphisms and mutations, copy number variations (CNVs) may play a substantial role in the pathogenesis of complex disorders. Here, we performed comparative genomic hybridization (CGH) to explore genome-wide CNVs in Wistar rats with a high susceptibility for the dopamine receptor agonist apomorphine (the so-called APO-SUS rats) and in their phenotypic counterparts (the so-called APO-UNSUS rats). APO-SUS rats show features as seen in patients with a complex aetiology and may thus be used to study (aspects of) complex disorder pathogenesis. Candidate genomic variations located in intragenic regions were validated and nine CNVs were found to segregate with both the original and the replicate APO-SUS/-UNSUS rat lines. The findings suggest a contribution of these CNVs to the complex APO-SUS phenotype.

Introduction

Twin, family and adoption studies have suggested that complex interactions at the genetic and environmental level underlie the aetiology of complex disorders, such as schizophrenia and bipolar disorder (Gottesman, 1991). Due to these interactions and the expected subtle influence of susceptibility genes, the genes and pathways for most complex disorders are however still unknown (van Loo and Martens, 2007). One approach to identify susceptibility genes and pathways for complex disorders is through the use of an animal model. In this study, we used the so-called APO-SUS/-UNSUS rat model, a model based on the differential response of the rats to the dopamine receptor agonist apomorphine (Cools et al., 1990). Apomorphine-susceptible (APO-SUS) and apomorphine-unsusceptible (APO-UNSUS) rat lines have been generated twice with a time interval of a decade (original lines in 1985 and replicate lines in 1995 (Cools et al., 1990; Ellenbroek et al., 2000)). Since the APO-SUS rats showed many features of psychopathology, with disturbances at the behavioural, physiological, endocrinological and pharmacological level similar to those seen in patients with a complex disorder (Ellenbroek and Cools, 2002), the APO-SUS/-UNSUS rat lines may represent an interesting model to study (aspects of) complex disorders.
Copy number variations (CNVs) may play a considerable role in the pathogenesis of complex disorders (Lee and Lupski, 2006). Estimates suggest that ~12% of the human genome consists of CNVs, ranging from kilobases to megabases in size (Redon et al., 2006). In the present study, we searched for CNVs in the genomes of the original as well as the replicate APO-SUS and –UNSUS rat lines, and wondered whether they are functionally linked to the complex APO-SUS phenotype. Since many human CNVs have been found to overlap with CNV regions in the rat (Guryev et al., 2008), findings in the rat may well be of significance for the human situation. To identify CNVs in the APO-SUS and –UNSUS rat genomes, we here used comparative genomic hybridisation (CGH), a technology that allows a genome-wide screening for CNVs with a relatively high resolution.

Materials and Methods

Experimental animals

The generation of the APO-SUS and -UNSUS rat lines with a high and a low susceptibility for apomorphine, respectively, has been described previously (Cools et al., 1990; Ellenbroek et al., 2000). The present experiments were performed with male APO-SUS and –UNSUS rats belonging to the 41st (original lines) and 26th (replicate lines) generation, and unselected male Wistar rats from the Nijmegen

outbred population. On post-natal day 60, the rats were injected with apomorphine (1.5 mg/kg s.c.) and gnawing scores were measured in a gnawing box for 45 minutes, as described previously (Cools et al., 1990). Following the measurements, the rats were sacrificed and the brains were isolated. All rats were bred and reared in the Central Animal Facility of the Radboud University (Nijmegen, The Netherlands) under approved animal protocols and in accordance with institutional guidelines.

CGH analysis

Genomic DNAs (gDNAs) from the replicate APO-SUS and –UNSUS rats were extracted from cortex tissue (DNeasy Blood and Tissue Kit, QIAGEN), randomly primed with Cyanine 3 or 5 and applied to the rat Agilent 244A arrays. Assays were performed in duplicate with dye swap. The arrays consisted of 235,000 probes (each ~60 nucleotides long), providing a mean resolution of one probe every 6 kb. Intensity ratios from the duplicate scans were averaged and normalized ratios were analysed with Agilent CGH analytics software (v3.4) to identify significant CNVs.

Genomic PCR and genomic quantitative PCR analysis

CNVs identified with the CGH technology were validated using genomic quantitative PCR analysis (qPCR) or standard PCR analysis (with an internal control to test for PCR efficiency). PCR assays were developed based on sequences from the UCSC Genome Browser database (UCSC Rn4; assembly Nov2004) with the PCR products spanning the CGH probe region. PCRs were performed in a total volume of 20 µl containing 10 ng gDNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.25% Nonidet P-40, 0.25% Tween-20, 250 µM each of the four deoxynucleotide triphosphates, 1 U *Taq* polymerase and 0.3 µM each of the four primers (one primer set specific for the chromosomal region in which the CNV was located (see Supplementary table for primer details) and a second primer set to test for PCR efficiency: 5'-CGTCTCCATCACGGGTTTG-3' and 5'-AAGTTTTCCCAGGAGCAGTCAA-3'). Reactions were carried out at 94°C for 60 sec, 60°C for 30 sec and 72°C for 45 sec for 35 cycles in a Peltier Thermal Cycler. Products were analysed on a 2% agarose gel. qPCR samples contained 10 ng gDNA, 1 x Power SYBR green PCR master mix (Applied Biosystems) and 0.3 µM of each primer (Biolegio; see Supplementary table for primer details) in a 10 µl volume. Reactions were carried out in a Rotor-Gene 6000 apparatus (Corbett Life Sciences) under the following conditions: 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Two genomic regions (complexin 1 on chromosome 14p22: 5'-AAGAGCAAACGTGGTGGGTAGA-3' and 5'-GCCTGGGCCACTCTCTTCA-3' and mineralocorticoid receptor on chromosome 19q11: 5'-GAAGTCTGCGTGGCGGG-3' and 5'-AAAAGAGGGTT

GTGAGGTCAATTTC-3') were amplified from all samples and used to validate the differences in copy number between the rats. qPCR data were analyzed with the Rotor-Gene 6000 software (v1.7).

Results

CNVs in the APO-SUS and –UNSUS rat genomes

To determine the number of CNVs in the APO-SUS/-UNSUS rat genomes and to identify their corresponding genomic regions, high resolution oligonucleotide CGH analysis on gDNAs from the replicate APO-SUS and –UNSUS rat lines was performed. Initially, 367 CGH probes (~0.16% of the Agilent CGH probes) were identified that represented differences in copy number between the APO-SUS and –UNSUS gDNAs. 201 Probes showed an increase in copy number and 166 a decrease in the APO-SUS genome compared to the APO-UNSUS genome. The CNVs were not equally distributed over the chromosomes, i.e. most of the 367 probes were located on chromosomes 4 and 20 (43 and 53 probes, respectively), whereas no probes were found on chromosome 19, only two on chromosome 18, three on chromosome 9, and four on chromosomes 6 and 15 (figure 1).

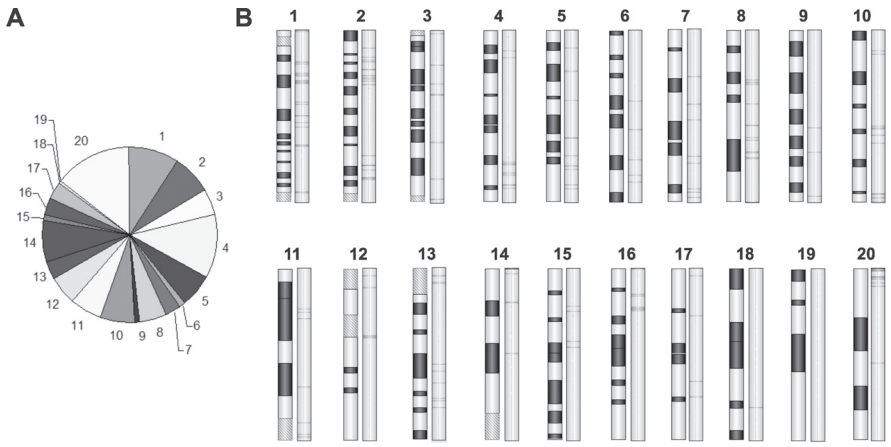

Figure 1. *Chromosomal distributions of CNVs within the APO-SUS and -UNSUS rat genomes. (A) CNVs were not equally distributed over the 20 autosomal chromosomes (the X and Y chromosomes were not included in the CGH analysis); no CNV was found on chromosome 19 and only two were present on chromosome 18. The largest number of CNVs was found on chromosomes 4 and 20. (B) Intrachromosomal distributions of the CNVs. Per chromosome two bars are shown: The first bars represent a schematic overview of the chromosomes, with the light-colored bands representing euchromatin and the dark-colored bands representing heterochromatin regions. The second bars illustrate the locations of the APO-SUS/-UNSUS CNVs.*

In our attempt to identify CNVs with a functional relevance, we decided to focus mainly on CNVs located within a gene(s). To validate the intragenic candidate regions (132 probes covering 47 CNVs; table 1), PCR or qPCR analysis was performed with gDNAs from eight APO-SUS rats with high gnawing scores (>500 counts/45 min) and eight APO-UNSUS rats with low gnawing scores (<10 counts/45 min). This analysis revealed 25 regions that contained a CNV that segregated with the APO-SUS/-UNSUS lines. Of these regions, 16 showed an increase in copy number and 9 a decrease in the APO-SUS compared to the APO–UNSUS genomes (table 2). Interestingly, all APO-UNSUS rat genomes examined showed similar CNV patterns, with only four of the 25 CNVs displaying individual differences within the group (n = 8). In contrast, the APO-SUS rat genomes showed more variations in their patterns with nine CNVs exhibiting individual differences (n = 8) (table 2).

Table 1. Intragenic CNVs found by APO-SUS and –UNSUS CGH analysis

S[a]	Region	Probe localisation[b]	Gene
+	1q12	48844408-48844467	afadin (Af6)
+	1q21	81322251-81346296	cytochrome P-450b type e
+	1q21	81355452-81355509	cytochrome P450e-L (Cyp450IIB2)
+	1q31	132513907-132513966	LRRGT00049
+	1q54	252177147-252177206	cyclin M2 (Cnnm2)
-	2q14	40418324-40418383	cAMP-specific phosphodiesterase PDE4D7 (Pde4d)
-	2q16	58321364-58321423	solute carrier family 1 (glial high affinity glutamate transporter), member 3 (Slc1a3)
-	2q16	58945286-58962650	prolactin receptor
+	3q12	34109106-34109165	rattus norvegicus cDNA sequence, complete 5' and 3' UTR's.
-	3q21	52825116-52825175	glutamic acid decarboxylase 1 (Gad1)
+	3q24	62659492-62659551	phosphodiesterase 1A, calmodulin-dependent (Pde1a)
-	3q42	146565477-146586794	RNA-binding region (RNP1, RRM) containing 2 (Rnpc2)
-	4q42	156513753-156513812	Ninjurin 2 (Ninj2)
+	4q42	167373472-167597397	LINE-1 retrotransposon nucleic acid binding protein mRNA
+	4q42	167610209-167713739	Ly49 inhibitory receptor 9 (Ly49i9)
+	4q42	167722626-167769236	LINE-1 retrotransposon nucleic acid binding protein mRNA
+	5q21	40864501-40864560	fucosyltransferase 9 (Fut9)
+	5q34	125542582-125542641	disabled homolog 1 (Drosophila) (Dab1)
+	5q36	135556739-135571102	cytochrome P450, 4a12 (Cyp4a12)
-	5q36	135620211-135646248	cytochrome P450, subfamily 4A, polypeptide 11 (Cyp4a11)
+	5q36	158440757-158440816	Ba1-651 mRNA

continued on next page

continued

S[a]	Region	Probe localisation[b]	Gene
+	7q35	133872643-133886266	nel-like 2 homolog (chicken) (Nell2)
+	8q21	37240112-37240171	urinary protein 2 (Rup2)
-	8q24	71093969-71129268	anterior pharynx defective 1b homolog (C. elegans) (Aph1b)
+	8q24	80216254-80216313	Mitogen-activated protein kinase 6 (Mapk6)
-	8q24	81962445-81974483	tubulointerstitial nephritis antigen (Tinag)
+	8q31	90466184-90466243	LRRGT00008 mRNA
-	10q24	63190604-63206723	Rabphilin 3A-like (without C2 domains) (Rph3al)
+	10q26	72239224-72239283	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52 (Ddx52)
+	10q32.2	105202914-105202973	CD300 antigen like family member F (Cd300lf)
-	10q32.3	107465582-107465641	septin 9 (Sept9), transcript variant 2
+	10q32.3	109397560-109397619	brain-specific angiogenesis inhibitor 1-associated protein 2 (Baiap2)
-	11q11	20602835-20602894	neural cell adhesion molecule 2 (Ncam2)
-	11q23	78379058-78415151	similar to LIM domain containing preferred translocation partner in lipoma (LOC288010)
-	11q23	84075431-84192014	clone LuMo47.3 immunoglobulin lambda light chain mRNA
-	13q21	66504082-66504141	niban protein (Niban)
+	13q24	90017140-90017199	Spectrin alpha 1 (Spna1)
-	14p21	21949892-21965334	sulfotransferase, estrogen preferring (Ste)
+	14p21	22155706-22170876	UDP glycosyltransferase 2 family, polypeptide B (Ugt2b)
-	17q12.1	72111961-72112005	GTP binding protein 4 (Gtpbp4)
+	20p12	2783283-2796035	MHC class I RT1.Aw3 protein, haplotype l.
+	20p12	3433631-3472185	clone:bC9, differentially expressed in pylorus.
+	20p12	3497512-3497571	dimethylarginine dimethylaminohydrolase 2 (Ddah2)
+	20p12	3499460-3612973	Chloride intracellular channel 1 (Clic1)
-	20p12	4700166-4700225	RT1 class II, locus Ba (RT1-Ba)
-	20p12	4733170-4733229	RT1 class II, locus Bb (RT1-Bb)
+	20p12	5810464-5810523	nudix (nucleotide diphosphate linked moiety X)-type motif 3 (Nudt3)

[a]S: CGH results of the APO-SUS (S) and -UNSUS (U) comparison. + : more copies in APO-SUS compared to -UNSUS, - : less copies in APO-SUS compared to -UNSUS. [b]Probe localisation is based on UCSC genome browser rat November 2004.

Since the APO-SUS and –UNSUS lines have been made twice via separate and independent selection and breeding procedures (original lines started in 1985 and replicate lines in 1995), similar representations of a particular CNV in the original and replicate APO-SUS and –UNSUS lines would point to a link between this CNV and the APO-SUS/-UNSUS phenotype. We therefore examined the original APO-

Table 2. Intragenic CNVs in APO-SUS and –UNSUS rats from the original and replicate lines

Gene	APO-UNSUS original line								APO-UNSUS replicate line								APO-SUS original line								APO-SUS replicate line							
Af6 *	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cyp450b type e *	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cyp450IIB2 *	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cnnm2 *	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	+	+	-	+	-	+	+
Pde4d	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
prolactin receptor	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pde1a	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Dab1 *	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+
Cyp4a12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Cyp4a11	±	-	-	±	-	±	±	±	±	±	±	±	+	+	+	+	±	+	±	±	±	±	-	±	-	-	-	-	±	±	±	+
Ba1-651	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Nell2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Rup2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aph-1b *	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cd300lf	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Baiap2 *	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Ncam2 *	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LOC288010	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	±	+	±	±	+	±	±
LuMo47.3	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	±	+	+	-	±	-
Niban	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Spna1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gtpbp4 *	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	-	-	+
RT1.Aw3	±	-	-	±	-	±	-	±	±	-	-	±	±	±	±	-	-	+	±	±	±	±	-	-	±	±	±	±	+	±	+	±
Clic1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	±
Nudt3	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*+: relatively high PCR signal; -: relatively low PCR signal; ±: intermediate PCR signal; *: similar distributions in the original and replicate lines.*

SUS and –UNSUS genomes for the 25 CNVs found to be different in the replicate lines. Interestingly, nine of the CNVs (CNVs located in afadin6 [AF6 on chromosome 1q12], cytochrome P-450b type e [Cyp450b type e; 1q21], cytochrome P450e-L [Cyp450IIB2; 1q21], cyclin M2 [Cnnm2; 1q54], disabled homolog 1 [Dab1; 5q34], anterior pharynx defective 1b [Aph-1b; 8q24], brain-specific angiogenesis inhibitor 1-associated protein 2 [Baiap2; 10q32.3], neural cell adhesion molecule 2 [Ncam2; 11q11] and GTP binding protein 4 [Gtpbp4; 17q12.1]) showed a similar distribution in the original and replicate lines (table 2).

Intragenic CNVs in the APO-SUS/-UNSUS rat genomes and apomorphine susceptibility

Next, we wondered whether any of the nine SUS/-UNSUS CNVs that were similarly distributed among the original and replicate lines would correlate with apomorphine susceptibility, the criterion originally used to create the APO-SUS and –UNSUS rat lines. For this purpose, we decided to crossbreed rats from the replicate APO-SUS line with rats from the replicate APO-UNSUS line. Following intercrossing of the offspring, males from the resulting F_6 generation were tested for their apomorphine susceptibility and eight rats with a low apomorphine susceptibility (<10 counts/45 min) and eight rats with a high apomorphine susceptibility (>500 counts/45 min) were selected for CNV analysis. None of the nine CNVs showed a correlation with apomorphine susceptibility, indicating that none of the CNVs was solely responsible for the susceptibility to the drug (table 3).

In addition, we analysed the nature of the nine SUS/-UNSUS CNVs in gDNAs from normal (unselected) Wistar rats from the Nijmegen outbred population. gDNAs from

Table 3. Intragenic CNVs in crossbred APO-SUS/-UNSUS rats with a low and high apomorphine susceptibility

Gene	L	L	L	L	L	L	L	L	H	H	H	H	H	H	H	H	U	S
Af6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Cyp450b type e	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+
Cyp450IIB2	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-	+
Cnnm2	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	-	+
Dab1	-	+	-	+	-	+	-	-	-	+	-	-	+	-	-	-	-	+
Aph-1b	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	-
Baiap2	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+
Ncam2	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-
Gtpbp4	-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-

L: APO-SUS/-UNSUS crossbred with a low apomorphine susceptibility; H: APO-SUS/-UNSUS crossbred with a high apomorphine susceptibility; U: APO-UNSUS; S: APO-SUS; +: relatively high PCR signal; -: relatively low PCR signal; ±: intermediate PCR signal.

eight Wistar rats with a low apomorphine susceptibility and from eight Wistar rats with a high apomorphine susceptibility were analysed. PCR and qPCR analyses of these 16 Wistar genomes revealed that again none of the nine CNVs was linked to apomorphine susceptibility (table 4).

Table 4. Intragenic CNVs in Wistar rats with a low and high apomorphine susceptibility

Gene	L	L	L	L	L	L	L	L	H	H	H	H	H	H	H	H	U	S
Af6	+	-	-	-	-	+	-	+	-	-	+	+	+	+	-	-	-	+
Cyp450b type e	+	-	-	+	+	+	-	+	-	+	-	+	+	+	+	+	-	+
Cyp450IIB2	+	+	-	+	+	+	-	+	-	+	-	+	+	+	+	+	-	+
Cnnm2	-	-	+	+	+	-	+	+	+	-	-	+	-	+	-	+	-	+
Dab1	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	+
Aph-1b	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Baiap2	+	+	+	+	+	-	+	+	-	+	+	+	-	+	+	+	-	+
Ncam2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Gtpbp4	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-

L: Wistar rat with a low apomorphine susceptibility; H: Wistar rat with a high apomorphine susceptibility; U: APO-UNSUS; S: APO-SUS; +: relatively high PCR signal; -: relatively low PCR signal; ±: intermediate PCR signal.

Discussion

In this study, we identified 25 CNVs that segregated with the phenotypically well-characterized replicate APO-SUS and –UNSUS rat lines. Interestingly, nine of the 25 CNVs were found in a similar distribution in both the original and replicate APO-SUS and –UNSUS lines, indicating that these CNVs may well be functionally linked to aspects of the complex APO-SUS phenotype. One of the CNVs, namely the CNV located within the *Aph-1b* locus, has been described previously as a gene-dosage imbalance correlating with (aspects of) the complex APO-SUS phenotype. Whereas APO-UNSUS rats harbour three gene copies of the γ-secretase component Aph-1b, APO-SUS rats have only one or two copies. This gene-dosage imbalance was due to an unequal crossing over event (nonallelic homologous recombination) between two direct repeats (a segmental duplication) within the *Aph-1b* locus (Coolen et al., 2005).

We now thus identified eight new CNV loci that may contribute to the complex APO-SUS phenotype, namely AF6, Cyp450b type e, Cyp450IIB2, Cnnm2, Dab1, Baiap2, Ncam2 and Gtpbp4. AF6, also known as myeloid/lymphoid or mixed-lineage leukemia translocated to 4 (MLLT4), is an actin filament-binding protein that regulates cell-cell adhesions. Since *AF6* knock-out mice show severe developmental

defects (e.g. disorganisation of the ectoderm, impaired mesoderm migration and loss of somites), AF6 is thought to be essential for proper morphogenesis (Ikeda et al., 1999). Two cytochrome P450 family genes contained a CNV. The many cytochrome P450 family members are involved in the metabolism of thousands of endogenous and exogenous compounds (Brown et al., 2008), and may thus influence multiple cellular processes. Cnnm2 is a protein with a still unknown function. Interestingly, Cnnm2 is located within a quantitative trait locus (QTL) for behavioural stress responses (Ahmadiyeh et al., 2005), a feature found to be different between the APO-SUS and -UNSUS rats (Rots et al., 1995). Dab1 is an adaptor protein that plays a key role in brain development. Dab1 mediates reelin signalling and regulates migration of cortical neurons in early brain development. In mice, reduced expression of Dab1 resulted in widespread misplacement of neurons and ataxia (Sheldon et al., 1997). In addition, within the genomic Dab1 region, a QTL for spike wave discharge intensities (Swd2; T2swd/wag) has been identified (Gauguier et al., 2004), a feature also affected in APO-SUS rats (de Bruin et al., 2000). Baiap2 functions as an insulin receptor tyrosine kinase substrate and may be involved in neurodegenerative diseases (Mackie and Aitken, 2005). Ncam2 belongs to the immunoglobulin superfamily and plays a role in olfactory sensory information projections (Alenius and Bohm, 1997). Finally, Gtpbp4 belongs to the family of GTPases involved in the regulation of a wide variety of processes during neuronal development, including neurite branching, axonal navigation and synapse formation. Thus, the eight newly identified chromosomal loci containing a CNV may well contribute to the complex APO-SUS phenotype. However, the functional consequence, if any, of these new CNVs for the APO-SUS and –UNSUS rat phenotypes is at present unclear.

Guryev et al. (2008) have reported that in CNV-harbouring regions within the genomes of the Brown Norway-Lx and SHR/OlaIpcv rat strains up to 44% of the genes were differentially expressed, indicating a functional effect for a substantial number of CNV-containing regions. Extensive spatio-temporal mRNA and protein expression profiling will reveal whether a CNV leads to aberrant expression levels of the respective genes in the APO-SUS/-UNSUS rat model. Whether any of such CNVs is indeed linked to the complex APO-SUS phenotype has to await time-consuming experiments based on crossing, backcrossing, genetic re-selection and subsequent phenotypical analyses of the selected APO-SUS/-UNSUS rat lines.

Since human and rat CNVs appear to be overlapping (Guryev et al., 2008), our findings may well be of significance for understanding complex human disorders. Interestingly, for six of the nine genes with a CNV in the replicate and original APO-SUS/–UNSUS rat genomes (AF6, Cyp450b type 2, Cyp450IIb2, Dab1, Baiap2 and Ncam2), CNVs have been reported in the corresponding human genes as well (based on Database of Genomic Variants; build 36, update 26 June 2008 (Iafrate et

al., 2004)). It would therefore be of interest to examine whether any of these human CNVs is associated with a complex disorder in human.

None of the CNVs in the nine APO-SUS/–UNSUS chromosomal regions was directly linked to apomorphine susceptibility in crossbred APO-SUS/-UNSUS or unselected Wistar rats. The lack of a direct link with any of the CNVs likely indicates that, in addition to environmental factors, genetic variations other than the CNVs identified here may contribute to the susceptibility for apomorphine. Furthermore, apomorphine susceptibility may not be influenced by only a single CNV (or another genetic variation), but rather by multiple genetic variations. Besides, in our quest to identify a correlation between a particular genomic region and apomorphine-susceptibility we focused on CNVs located within or nearby a gene. A CNV located in an intergenic region may however also have a functional effect, e.g. through regulatory elements present within a CNV region.

Most likely our study did not reveal all CNVs that occur in the APO-SUS and –UNSUS rat genomes. The Brown Norway genome has been sequenced and represents the rat genome deposited in genomic assemblies and databases, and has been chosen for constructing the CGH array. Yet, the Brown Norway genome is known to be genetically different from the genome of the Wistar rat (Saar et al., 2008), the strain used to create the APO-SUS and –UNSUS lines. Wistar-specific genomic segments are therefore not represented on the genomic arrays and thus additional CNVs may exist in this species. Nevertheless, our results show that at least nine CNVs are present in the APO-SUS/-UNSUS genomes and may play a role in the complex APO-SUS phenotype.

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Supplemental table. Primer sequences used for PCR and qPCR validation of CGH results

Gene	5'-sequence-3'	amplicon size (bp)	PCR or qPCR validation
Af6	5'-TCGTAGTGCCTACGATTGAAATAGC-3' 5'-GTCATTGGTTCACTTAAAAAAAAAAAAA-3	130	qPCR
Cyp450b type e	5'-CCTTTTTGTGTTGTCAGTAGCCC-3' 5'-AAATCAGGTGTCAAGCCCAGTC-3'	187	PCR
Cyp450IIB2	5'-TCTTAACTAATGGAGCATCCTGGTC-3' 5'-TGGCATGAATATCATCTTACCCC-3'	104	qPCR
Cnnm2	5'-GTTGCTGGATACTAATCAAACTGTTTG-3' 5'-CATTAGTGACAGGGCATTGGTC-3'	101	PCR
Pde4d	5'-CAGATGGGAGAAGAGTTAAAGAGTCTTT-3' 5'-CCTTCTCTATCAGGAAATTTACTACCAAA-3'	110	PCR
prolactin receptor	5'-TATTCTAGTTTTACAAGTTGAAAGTTTATCCAC-3' 5'-CATCAAGAACTCCATTTGTTACTAAGTCTG-3'	100	PCR
Pde1a	5'-CACAATGTTACTATCATAAGGAACCATTT-3' 5'-GCTGTAGATAATTAATAAGGTGACAGATGTTA-3'	112	PCR
Dab1	5'-AGAAATGCAAAAGTTAAAAAGGATACATC-3' 5'-CAAAACTACCCCCAAGACAACC-3'	101	PCR
Cyp4a12	5'-ATTTAAAAAATTACAACTCTTCTCCTGGTT-3' 5'-GGTTAAAATAGATCTGAGTTCAAACGTATC-3'	115	qPCR
Cyp4a11	5'-ATGATATGTAGGCTTCAAGTGTTAGCG-3' 5'-TTTTTTACCCTTTCAACCCTCAAA-3'	151	qPCR
Ba1-651	5'-CTCTGCAGGTCCTAGCCTCCTA-3' 5'-AATACACACTGGACCTACTTACAATGGTA-3'	102	PCR
Nell2	5'-AGCCTTAGTAATTCACGCACCATT-3" 5'-GGAGAGAGAAAAGCTGAAGAGTAGTCA-3'	100	PCR
Rup2	5'-AATCGATTCTAGAGGAATTTGTCGG-3' 5'-GAGTGGTTGCTACAACCCAGG-3'	91	PCR
Aph-1b	5'-GAGTGGGAATGAACTAGCCCT-3' 5'-GTCCTCTAAACTGTATTCCTGCTGG-3'	109	qPCR
Cd300lf	5'-TGTGTGATGTGTGGGTGGAAT-3' 5'-AGTATGAGGCAGAATAGAACAGGGTAAA-3'	107	PCR
Baiap2	5'-AAGGTGTTGGTTACTAGAGAGCCTT-3' 5'-TGGCAACCCTCACTGAGTAGGT-3'	109	PCR
Ncam2	5'-TCATTCATTTATAAGCGATGCCA-3' 5'-TTAACACAAAATGAATGCTAGATATCAAAAA-3'	112	qPCR
LOC288010	5'-CCGCAATTCTGCACAATCAT-3' 5'-AGCGGCTTGTACATGCGAG-3'	101	qPCR
LuMo47.3	5'-AATTATTGAGGATTGTTGATTTTGAAGTT-3' 5'-GACTCACATTGTGCACTTTGAAAAC-3'	143	PCR
Niban	5'-AATTAGGGATGTTTGGTCTTTATGGTTA-3' 5'-ATCCGCTGAAAAGTAAAAGCTGA-3'	117	PCR
Spna1	5'-TACTTACTACTAAATCTTGTGTGTCCCCTT-3' 5'-ATTTCCAGAACTATGAGTTTACTTAATAGTGAA-3'	101	PCR
Gtpbp4	5'-GCCCTTCTTTGCAGGCTTC-3' 5'-AAAGTGAAACCCGCGTGTG-3'	103	qPCR
RT1.Aw3	5'-ACGACCCGTTCAATCTGTCC-3' 5'-TTGCAAATCCTATTTAGCTGTGAGA-3'	101	qPCR
Clic1	5'-GTCTTGACCTTCGACCCTGG-3' 5'-CAAGGGACACGAATGGAGTTTC-3'	99	PCR
Nudt3	5'-CGACACTCGTGGCTTACAGAGATA-3' 5'-TGGTCTTAGGGTTACTGTTTCTTGTAGTTAT-3'	140	PCR

Part B
Human

Chapter B1

Male-specific association between a γ-secretase polymorphism and premature coronary atherosclerosis

With
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Abstract

Atherosclerosis is a common multifactorial disease resulting from an interaction between susceptibility genes and environmental factors. The causative genes that contribute to atherosclerosis are elusive. Based on recent findings with a Wistar rat model, we speculated that the γ-secretase pathway may be associated with atherosclerosis. We have tested for association of premature coronary atherosclerosis with a non-synonymous single-nucleotide polymorphism (SNP) in the γ-secretase component APH1B (Phe217Leu; rs1047552), a SNP previously linked to Alzheimer's disease. Analysis of a Dutch Caucasian cohort (780 cases; 1414 controls) showed a higher prevalence of the risk allele in the patients (odds ratio (OR) = 1.35), albeit not statistically different from the control population. Intriguingly, after gender stratification, the difference was significant in males (OR = 1.63; p = 0.033), but not in females (OR=0.50; p=0.20). Since Phe217Leu-mutated APH1B showed reduced γ-secretase activity in mouse embryonic fibroblasts, the genetic variation is likely functional. We conclude that, in a male-specific manner, disturbed γ-secretase signalling may play a role in the susceptibility for premature coronary atherosclerosis.

Introduction

Atherosclerosis is the basis of coronary artery disease and thought to be a multifactorial disease caused by susceptibility genes that act in concert with environmental factors. A number of susceptibility genes have been identified (e.g. apolipoprotein E (*APOE*) (Horejsi and Ceska, 2000), low density lipoprotein receptor (Salazar et al., 2000) and methylenetetrahydrofolate reductase (Morita et al., 1997)), but the signalling pathways responsible for vascular cell pathology are elusive. Interestingly, Wistar rats that display a high susceptibility for the dopamine receptor agonist apomorphine, the so-called apomorphine-susceptible (APO-SUS) rats (Cools et al., 1990; Ellenbroek et al., 2000), show an impaired vasorelaxation to adrenergic stimuli when compared with their phenotypic counterparts APO-UNSUS rats (Smits et al., 2002; Riksen et al., 2003). Impaired vasorelaxation is associated with an increased risk for the development of hypertension and vascular diseases such as atherosclerosis (Hadi et al., 2005). We recently identified a gene-dosage imbalance of the γ-secretase component Aph-1b as the molecular-genetic basis of the difference between the APO-SUS and -UNSUS rats (Coolen et al., 2005). The γ-secretase enzyme complex cleaves many type I transmembrane proteins, including the amyloid-β (Aβ) precursor protein APP (known to be involved in neuronal amyloid plaque formation in Alzheimer's disease [AD]), NOTCH1-4, neuregulin, low-density lipoprotein receptor related protein (LRP1, 2 and 8) and N-cadherin (Koo and Kopan, 2004; Kopan and Ilagan, 2004).
In view of the above findings, we hypothesize that the γ-secretase pathway may be linked to atherosclerosis. Increasing evidence suggests a link between altered vascular homeostasis, as seen in atherosclerosis, and the neurodegenerative disease AD. Apart from a partially overlapping epidemiology and an altered cholesterol homeostasis, atherosclerosis and AD have also been found to share genetic risk factors, including *APOE* and *LRP1* (Kang et al., 1997; Rubinsztein and Easton, 1999; Horejsi and Ceska, 2000; Schulz et al., 2002). Since the rare non-synonymous single-nucleotide polymorphism (SNP) Phe217Leu (rs1047552; T>G) in the human *APH1B* gene has recently been found to be associated with AD (Poli et al., 2008), we have now tested whether this SNP is also associated with premature coronary atherosclerosis.

Materials and Methods

Subjects

We selected consecutive Dutch premature coronary atherosclerosis patients (n=780, age 43.3 ± 5.3; 582 males, age 43.6 ± 5.3 and 198 females, age 42.5 ± 5.5) who qualified for inclusion after a myocardial infarction, surgical or percutaneous coronary revascularization, or a coronary angiogram with evidence of at least a

70% stenosis in a major epicardial artery (Atherosclerosis Outpatient Clinic of the Academic Medical Center of the University of Amsterdam). The study was approved by the Medical Ethical Committee of the Academic Medical Center (Amsterdam, The Netherlands) and all patients gave written informed consent. The control subjects (n=1414, age 51.8 ± 11.9; 938 males, age 53.7 ± 10.9 and 476 females, age 48.2 ± 12.9) were from the Sanquin Blood Bank. Volunteers were recruited at their blood donation session at one of the collection sites of the Sanquin Blood Bank covering the north west of the Netherlands. Therefore more than >95% of these donors lived in the Dutch postal code area 1000-4000. This area was chosen while the Amsterdam patient cohort geographically overlaps the region of the blood donor cohort serving as control donors.

Biochemical analysis

In the atherosclerosis patients, plasma cholesterol and triglycerides were determined with commercially available enzymatic methods (Boehringer Mannheim, FRG, No. 237574, and Sera-PAK, No. 6639, respectively). To determine high-density lipoprotein cholesterol, the polyethylene glycol 6000 precipitation method was used. Low-density lipoprotein cholesterol was calculated by the Friedewald formula.

APH1B genotyping

Following the isolation of genomic DNA, *APH1B* (MIM# 607630) Phe217Leu genotyping was performed via allele-specific PCR using primers specific for SNP rs1047552 (outer/general primers: forward: 5'-TGCCTTCTAGGGTTACCATCTGA-3' and reverse: 5'-AGTCGGCTTTACACTGTCCCA-3'. Inner/specific primers: forward specific for the "T-allele": 5'-AATAAACCTGGCGTCAGCATTT-3' and reverse specific for the "G-allele": 5'-GCCCATGAGCACCAGGATTATC-3').

Generation of Aph-1abc$^{-/-}$ mouse embryonic fibroblast (MEF) lines

Conditionally targeted (*Aph-1a* and *Aph-1c*) or classically targeted (*Aph-1b*) mice were described before (Serneels et al., 2005). Animals carrying a null allele were obtained after breeding with transgenic mice expressing a *Pgk* driven Cre-recombinase. Mouse embryos were dissected at E8.5 from *Aph-1abc*$^{+/-}$ crosses and genotype was determined by PCR analysis on yolk sacs. Mouse embryonic fibroblast (MEF) cultures were derived from dissociated *Aph-1abc*$^{-/-}$ mouse embryos (Herreman et al., 2003).

Generation of stable cell lines

The Phe217 to Leu217 mutation was made using the QuikChange II site-directed

mutagenesis kit (Stratagene), using human wild-type APH1B cloned into pcDNA3.1 Zeo+ (Invitrogen) as template. The primers used to introduce the mutation were: 5' -CCTGGCGTCAGCATTGATAATCCTGGTGCTC-3' (forward); 5'-GAGCACCA GGATTATCAATGCTGACGCCAGG-3' (reverse). Phe217 and Leu217 hAPH1B were recloned into pMSCVpuro* and cotransfected into HEK293 cells with helper plasmid pIK Ecopac for packaging into retroviruses. Retroviruses were harvested and snap frozen aliquots were stored at –80°C until use. MEF *Aph-1abc*$^{-/-}$ cells were transduced with retrovirus for 24 hrs followed by puromycin selection in DMEM/ F12 supplemented with 10% FCS until stable lines were obtained.

Measurement of gamma-secretase activity towards different substrates

Stably transfected MEFs were seeded and grown to confluency. Cells were rinsed twice with ice-cold PBS and lysed in 1% Triton, and postnuclear fractions were isolated by centrifugation at 10,000g for 15 min at 4°C. Proteins were quantified using a standard Bradford assay (Pierce) and 10-15 µg protein/lane was loaded on Bis-Tris SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes for Western blot detection for the indicated proteins. Gamma-secretase activity towards each substrate was expressed as the level of substrate C-terminal fragment (the direct gamma-secretase substrate) relative to levels of full-length protein. For densitometric quantification, the films were scanned using an Image Scanner (Amersham Pharmacia) and analyzed using ImageMaster.

Antibodies

APP was detected with C-terminal pAb B63.1 and syndecan 3 with C-terminal mAb 2E9. An antibody against the N-cadherin C-terminus (clone 32, BD Bioscience) was purchased.

Statistical analysis

Genotype frequencies were tested for the Hardy-Weinberg equilibrium. Differences between cases and controls were analysed by standard contingency table analysis using two-tailed χ^2 test probabilities. Odds ratios (95% confidence intervals (CI)) were calculated as an index of the association of the *APH1B* genotypes with premature atherosclerosis. Continuous and categorical biochemical and clinical variables were determined with the Student's t-test and χ^2-analysis, respectively. A p-value < 0.05 was considered statistically significant (GraphPad Software Inc, San Diego, CA, USA). Though being conservative, Bonferonni correction was used to determine the significance of the biochemical and clinical variables. Power calculations were estimated using Quanto v1.2 (Gauderman, 2002).

Results

Male-specific association of APH1B Phe217Leu with premature coronary atherosclerosis

Since a gene-dosage imbalance of the *Aph-1b* gene was the molecular-genetic basis of the APO-SUS/-UNSUS rat model (Coolen et al., 2005) and the model was characterized by a disturbed endothelium-dependent vascular reactivity (Smits et al., 2002; Riksen et al., 2003), we tested the hypothesis that a genetic variation in the *APH1B* gene may contribute to atherosclerosis susceptibility in humans. In a Dutch case-control cohort consisting of 780 patients with premature coronary atherosclerosis and 1414 controls, we found a higher prevalence of the risk allele (G-allele) in the patients, albeit not statistically different ($\chi^2 = 2.09$, $df = 1$, p =0.15; OR = 1.35; CI = 0.90-2.01). Intriguingly, after gender stratification, the difference was significant in the male population ($\chi^2 = 4.52$, $df = 1$, p =0.033; OR = 1.63; CI = 1.04-2.58), whereas females were not significantly different ($\chi^2 = 1.62$, $df = 1$, p = 0.20; OR = 0.50; CI = 0.17-1.48) (table 1). Power analysis showed that for the detection in the female subpopulation of a risk effect similar to that observed in the male subpopulation, the power was insufficient (92% power for the total population; 81% power for the male subpopulation and 38% power for the female subpopulation, assuming a relative risk of 1.63, a disease allele frequency of 3.3% and a disease prevalence of 5%). All genotype distributions tested (cases and controls) fulfilled the Hardy-Weinberg criteria (data not shown).

Table 1. Genotype and allele frequencies for the *APH1B* Phe217Leu variation in a Dutch case-control study on premature coronary atherosclerosis

		Genotype Frequencies (%)			Allele Frequencies (%)			
	n	TT	TG	GG	T	G	*p*	OR
total								
controls	1414	96.0	4.0	0	98.0	2.0		
patients	780	94.6	5.4	0	97.3	2.7	0.15	1.35
males								
controls	938	95.9	4.1	0	98.0	2.0		
patients	582	93.5	6.5	0	96.7	3.3	0.033*	1.63
females								
controls	476	96.0	4.0	0	98.0	2.0		
patients	198	98.0	2.0	0	99.0	1.0	0.20	0.50

*Standard Chi-square tests were applied to evaluate the association with premature coronary atherosclerosis. OR = odds ratio; *p<0.05.*

Association of APH1B 217Leu with fibrinogen levels in premature coronary atherosclerosis

We then compared the association of the *APH1B* 217Leu allele with clinical parameters in the atherosclerosis patients, including the presence of risk factors (e.g. smoking behavior and occurrence of hypertension and diabetes mellitus), and the blood levels of lipid compounds (e.g. cholesterol, triglycerides, and low- and high-density lipoprotein cholesterol) (for a detailed overview of the parameters tested, see table 2). These parameters were not related to the *APH1B* Phe217Leu variation (table 2), except for a significant association ($p = 0.028$) with the fibrinogen levels in patients containing or lacking the *APH1B* Leu217 allele. Patients without the Leu217 allele displayed fibrinogen levels of 322.8 ± 80.55 gr/l ($n = 327$, plus SD), whereas patients with the Leu217 allele had levels of 375.0 ± 82.08 gr/l ($n = 12$, plus SD) (table 2); due to low female patient numbers, gender stratification for fibrinogen levels was not possible. After Bonferroni adjustment for multiple comparisons, however, no statistically significant association of the fibrinogen levels with the Leu217 allele was detected (Bonferroni's adjustment requires a significance level of $p \leq 0.00156$).

Evolutionary conservation of amino acid residue Phe217 within the APH1 family

The degree of conservation of an amino acid within a protein family is usually indicative of its importance for protein functioning. A multiple sequence alignment of members of the *APH1* family (figure 1) showed that the Phe217 residue is conserved from plant, invertebrates, lower vertebrates, rodents and primates to man. The various APH1 proteins all contain at residue 217 either a phenylalanine (F) or the conservative change to tyrosine (Y). The Support Vector Machine (SVM) score (http://www.SNPs3D.org) (Yue et al., 2006) of –1.12 for Phe217Leu indicates a likely impact of this substitution on APH1B protein function.

Functional analysis of the APH1B Phe217Leu polymorphism

We wondered whether the presence of a leucine instead of the conserved residue Phe217 of the APH1B protein would be of functional importance. *Aph-1abc*$^{-/-}$ mouse embryonic fibroblasts were stably transfected with human APH1B Phe217 or Leu217. γ-Secretase activity was measured by quantifying the levels of different γ-secretase substrates in cell culture extracts. We observed a 1.6-fold reduction ($p<0.05$, $n=8$) of γ-secretase activity towards one of its substrates, syndecan-3 (Schulz et al., 2003), indicating a subtle influence on γ-secretase cleavage activity. The cleavages of two other substrates, N-cadherin and APP, were slightly but not significantly changed (figure 2). Thus, in a substrate-dependent manner the Phe217Leu substitution affected γ-secretase cleavage activity.

Table 2. Clinical and biochemical characteristics of the premature coronary atherosclerosis patients with and without the *APH1B* 217Leu allele

	without *APH1B* 217Leu		**with *APH1B* 217Leu**		
	values ± SD	**n**	**values ± SD**	**n**	***p***
Age (years)	43.3 ± 5.3	739	42.9 ± 5.3	42	0.60
BMI (kg/m2)	26.9 ± 4.2	739	26.8 ± 3.1	42	0.88
Age first manisfestation vascular event (yrs)	41.6 ± 5.9	739	40.9 ± 6.1	42	0.47
Systolic blood pressure (mmHg)	129.1 ± 17.2	739	127.0 ± 18.5	42	0.45
Diastolic blood pressure (mmHg)	79.5 ± 10.5	739	81.2 ± 12.6	42	0.32
Total cholesterol prior to medication (mmol/l)	6.3 ± 1.9	286	6.6 ± 1.4	20	0.50
LDL-cholesterol prior to medication (mmol/l)	3.9 ± 1.3	220	4.4 ± 1.3	14	0.20
HDL-cholesterol prior to medication (mmol/l)	1.1 ± 0.4	231	1.0 ± 0.2	15	0.43
Triglycerids prior to medication (mmol/l)	1.8 ± 1.2	229	1.9 ± 0.7	15	0.77
Total cholesterol with medication (mmol/l)	5.0 ± 1.5	738	4.9 ± 1.7	42	0.88
LDL-cholesterol with medication (mmol/l)	3.0 ± 1.2	730	3.1 ± 1.8	41	0.78
HDL-cholesterol with medication (mmol/l)	1.1 ± 0.3	737	1.1 ± 0.4	42	0.41
Triglycerids with medication (mmol/l)	2.1 ± 4.0	738	1.8 ± 1.6	42	0.68
Apo A1 lipoprotein (mmol/l)	1.3 ± 0.3	495	1.2 ± 0.2	29	0.12
Apo B100 lipoprotein (mmol/l)	1.1 ± 0.6	494	1.0 ± 0.4	29	0.40
Lipoprotein (a) (mg/l)	215.2 ± 309.4	496	240.7 ± 377.1	30	0.66
Blood sedimentation rate (BSE) (mm/h)	10.4 ± 11.3	345	9.4 ± 5.2	16	0.74
Fibrinogen (gr/l)	322.8 ± 80.5	327	375.0 ± 82.1	12	0.028 *
	%	**n**	**%**	**n**	**p**
Acute myocardial infarction	73.34	739	69.05	42	0.54
Coronary angiography abnormal	98.23	621	97.14	35	0.64
Percutaneous transluminal coronary angioplasty	66.98	739	80.95	42	0.06
Coronary artery bypass grafting	7.98	739	4.76	42	0.45
Complaints claudication (<500 m walking distance)	11.77	739	7.14	42	0.36
Percutaneous transluminal angioplasty	7.58	739	7.14	42	0.92
Peripheral artery bypass grafting	2.84	739	0	42	0.27
Cerebro vascular accident	2.98	739	4.88	41	0.50
Smoking before the first event date	75.24	739	66.67	42	0.21
Treated for hypertension	25.44	735	33.33	42	0.26
Treated for diabetes mellitus	7.44	739	4.76	42	0.52
Hypercholesterolemia 1st degree relative	48.37	552	48.57	35	0.98
Hypertension treatment first degree relative	45.71	617	47.37	38	0.84
Diabetes treatment first degree relative	27.42	660	23.08	39	0.55

*Values are given as mean levels ± SD or as percentages. N, number of individuals tested; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; Apo, apolipoprotein. *p<0.05.*

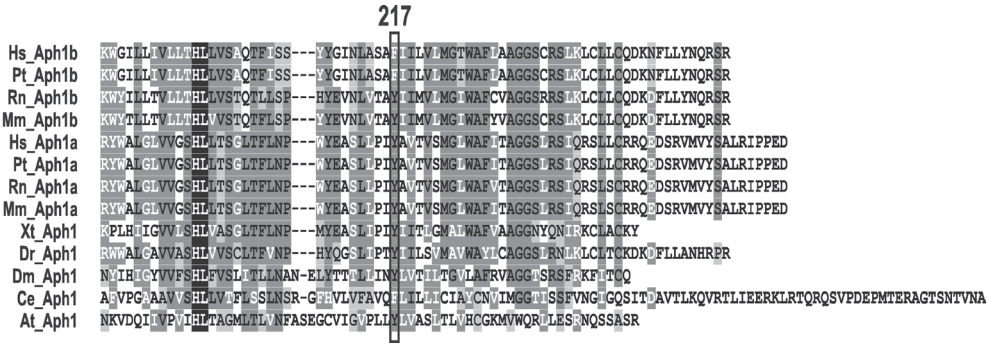

Figure 1. *Alignment of vertebrate and invertebrate amino acid sequences of the region within APH1 surrounding residue 217. A black background indicates identical amino acid residues; a grey background indicates a conservative amino acid change. Abbreviations: Hs, Homo sapiens; Pt, Pan troglodytes; Rn, Rattus norvegicus; Mm, Mus musculus; Xt, Xenopus tropicalis; Dr, Danio rerio; Dm, Drosophila melanogaster; Ce, Caenorhabditis elegans; At, Arabidopsis thaliana. Sequences were aligned using Vector NTI (9.0) with default parameters.*

Discussion

In this study we show that the non-synonymous Phe217Leu polymorphism in the human *APH1B* gene is a male-specific risk factor for premature coronary atherosclerosis. The reduced γ-secretase cleavage activity of Leu217 APH1B, albeit in a substrate-specific manner, suggests a functional relevance of this polymorphism. Functional consequences of reduced gene dosage of *Aph-1b* were also observed in the APO-SUS/-UNSUS rat model (Coolen et al., 2005), an animal model with complex features, including an impaired vasorelaxation response to adrenergic stimuli (Smits et al., 2002; Riksen et al., 2003), which increases the risk for the development of hypertension and vascular diseases. The gene-dosage imbalance of the *Aph-1b* gene (three gene copies in APO-UNSUS rats and one or two gene copies in APO-SUS rats) segregated with differences in γ-secretase cleavage activity and a number of other phenotypic characteristics (Coolen et al., 2005). We therefore speculate that a subtle effect on *APH1B* expression or function (copy number variation in rat or Phe217Leu variation in human, respectively) may affect γ-secretase activity and signalling downstream of gamma-secretase cleavage and, consequently, give rise to vascular complications. Interestingly, the *APH1B* Phe217Leu polymorphism was recently found to be also associated with AD (Poli et al., 2008). AD pathogenesis is frequently characterized by a peripheral vascular disturbance and is often linked to other cholesterol/lipoprotein diseases, including atherosclerosis (Casserly and Topol, 2004). In addition, cerebral atherosclerosis has been found to be a strong contributory factor to sporadic AD pathogenesis (Roher et al., 2003). The *APH1B*

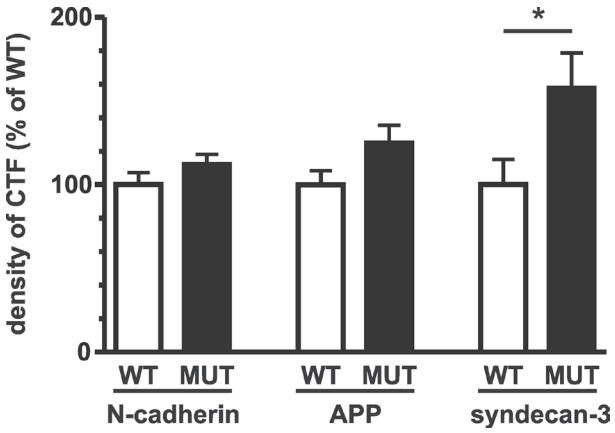

Figure 2. *γ-Secretase cleavage activity of human wild-type APH1B (Phe217) and human mutant APH1B (Leu217) stably transfected into Aph-1abc$^{-/-}$ mouse embryonic fibroblast cells. The levels of the C-terminal fragments (CTFs) of N-cadherin, APP and syndecan-3 were analysed in cells stably transfected with human APH1B Phe217 (WT) or human APH1B Leu217 (MUT). In the MUT cells, the CTF levels of N-cadherin and APP were slightly increased (1.1-fold and 1.2-fold, respectively), but the difference failed to reach significance. The levels of syndecan-3-CTF were significantly increased (1.6-fold; p=0.044), indicating a reduced γ-secretase cleavage activity. Bars represent quantifications of CTF signals normalized to full-length protein levels in eight stably transfected cell lines with the average level in WT cell lines set to 100%.*: p<0.05.*

Phe217Leu variation may thus play a dual role by affecting atherosclerosis as well as AD pathogenesis, suggesting that the two diseases have converged and that the γ-secretase signalling pathway is a susceptibility pathway for vascular complications.

In line with the above supposition, a number of the γ-secretase substrates have been implicated in vascular pathogenesis. *LRP,* which belongs to a gene family involved in mediating cellular uptake of cholesterol-rich lipoproteins (the low-density lipoprotein receptor (*LDLR*) gene family), is highly expressed in atherosclerotic lesions (Luoma et al., 1994; Hiltunen et al., 1998) and has been shown to represent a susceptibility gene for atherosclerosis (Schulz et al., 2002). The AD-associated γ-secretase substrate APP may be involved in vascular pathogenesis as well, since it metabolises cholesterol (Nelson and Alkon, 2005), physically interacts with LRP1 (Rebeck et al., 2001), and gives rise to the amyloid plaque constituents Aβ-40 and -42. Besides these well-studied substrates, the γ-secretase substrates NOTCH3, colony-stimulating factor 1 (CSF1), CD44, neuregulin and ERBB4 may also be involved in the vascular complications in patients with the *APH1B* Phe217Leu variation. Mutations in *NOTCH3* may cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoecephelopathy (CADASIL), a syndrome characterized

by systemic vascular smooth muscle cell (VSMC) degeneration (Wang et al., 2008). CSF1 can contribute to atherosclerosis development via fatty streak formation and progression to complex fibrous lesions (Rajavashisth et al., 1998), and CD44 may enhance atherosclerosis pathogenesis via reactive oxygen species (Vendrov et al., 2006), whereas neuregulin and ERBB4 are necessary for vascular growth and development (Gassmann et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996).

Our results show that, remarkably, only male individuals with atherosclerosis seem to be associated with the *APH1B* Phe217Leu variation. This might indicate a role for hormones or the involvement of a Y-chromosome-linked modulation. Such a gender-specific association has been observed for other SNPs as well, like a male-specific association of the *APOE2* and *APOE4* alleles with cardiovascular disease (Lahoz et al., 2001). Thus, susceptibility to many common diseases may well be the result of complex interactions involving gender, genes and environmental factors. Following Bonferroni correction for multiple testing, no significant associations between the *APH1B* polymorphism and plasma lipoprotein parameters or other risk factors were observed in the atherosclerosis patients. More detailed studies will be necessary to establish the biochemical mechanisms underlying atherosclerosis in patients carrying the Phe217Leu polymorphism.

In the present study, association was only tested for premature coronary atherosclerosis, a disease with a substantial heritability (Rissanen, 1979). To investigate whether the Phe217Leu SNP constitutes a susceptibility factor for other vascular complications, it would be of interest to also test for association in elderly atherosclerotic patients and in patients with other vascular defects (e.g. myocardial infarction). No significant association of the *APH1B* chromosomal region with coronary artery disease, myocardial infarction or other related diseases has been found in recent genome-wide association studies (GWAS) (Helgadottir et al., 2007; McPherson et al., 2007; O'Donnell et al., 2007; Samani et al., 2007). Thus, apart from the functional Phe217Leu polymorphism, the contribution of any additional genetic variant in the *APH1B* gene to the phenotype is expected to be small. Unfortunately, no results for the *APH1B* Phe217Leu polymorphism are available from the GWAS since this polymorphism was not represented on the arrays used in these studies. In addition, based on the data from the International HapMap-CEU project none of the SNPs tested in the GWAS is in near perfect proxy ($r^2 \geq 0.8$) to the Phe217Leu polymorphism. Therefore, the GWAS data do not provide any additional information concerning the functional *APH1B* Phe217Leu SNP.

In conclusion, our results suggest that the γ-secretase pathway is a candidate pathway for premature coronary atherosclerosis and warrant further studies on genetic variations in this pathway in various diseases with vascular complications.

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Chapter B2

Susceptibility for epilepsy and the γ-secretase pathway

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Submitted

Abstract

Epilepsy is a heterogeneous disorder with susceptibility genes and environmental factors that can act together to produce the phenotype. Based on our recent findings with a Wistar rat model, we hypothesized that the γ-secretase pathway may be involved in epileptic seizures. Here, we examined in Caucasian epilepsy-control cohorts the functional single-nucleotide polymorphism (SNP) Phe217Leu (rs1047552) in the human gene encoding the γ-secretase component APH1B. We find that the polymorphism was significantly associated ($\chi^2 = 3.90$, $df = 1$, $p = 0.048$; OR = 2.42; CI = 0.98-5.99) in a Dutch cohort of idiopathic generalized epilepsy (IGE), with a higher prevalence of the risk allele in the cases. Analysis of the polymorphism in an independent second IGE cohort from the United Kingdom (Kent) also showed a higher prevalence of the risk allele in the cases, albeit not statistically different from the control population ($\chi^2 = 0.21$, $df = 1$, $p = 0.65$; OR = 1.19; CI = 0.56-2.53). Furthermore, analysis in yet another independent group of epilepsy patients from the United Kingdom (Glasgow) showed a higher prevalence of the risk allele in the IGE than the non-IGE patients. Thus, the γ-secretase pathway may constitute a novel susceptibility pathway for IGE.

Introduction

Epilepsy is characterized by abnormal electrical brain activity and has a genetically determined aetiology. Numerous linkage and association studies have been performed to identify epilepsy susceptibility chromosomal regions and genes. Although a number of genes have been found to be linked to the familial monogenic epilepsies, consistent identification of the predisposing genetic variants in the more common epilepsies (e.g. idiopathic generalized epilepsies (IGEs)) has mostly not been achieved (reviewed by Dibbens et al., 2007). The expected subtle influence of susceptibility genes and the complex interactions with environmental factors increase the difficulty to unravel the aetiology of the common or complex epilepsies.

The findings reported here have their roots in our recent studies on the molecular genetic basis of a Wistar rat model with a high susceptibility to the dopamine receptor agonist apomorphine (so-called APO-SUS rats) (Cools et al., 1990). These rats display features resembling those of epilepsy, including abnormalities in brain information processing and increased bursts of bilateral synchronous spike wave discharges recorded with electroencephalography (Cools and Peeters, 1992; Ellenbroek et al., 1995; de Bruin et al., 2000). RNA expression studies and genetic analyses have revealed that the difference underlying the APO-SUS and its counterpart the APO-UNSUS rats concerns a gene-dosage imbalance of *Aph-1b* (Coolen et al., 2005), a component of the γ-secretase complex. The γ-secretase enzyme complex consists of four different integral membrane proteins, namely presenilin (either PS1 or PS2), nicastrin, presenilin-enhancer 2 (PEN2) and APH1 (Fortini, 2002; Francis et al., 2002; Goutte et al., 2002; Kimberly and Wolfe, 2003). The enzyme is responsible for the proteolytic processing of a wide variety of type I transmembrane proteins, including amyloid-β precursor protein (APP) and its relatives the APP-like proteins APLP1 and APLP2, Notch1-4, glutamate receptor 3 (GluR3), human leucocyte antigen-A2 (HLA-A2) and the voltage-gated sodium channel beta 2 subunit (Scn2b) (reviewed by Parks and Curtis, 2007). The reduction of *Aph-1b* gene copies in the APO-SUS rats segregated with a reduced tissue-specific and spatio-temporal γ-secretase cleavage activity (Coolen et al., 2006a; Coolen et al., 2006b).

On the basis of the results obtained with the APO-SUS and -UNSUS rat model, we speculated that a genetic variation in the *APH1B* gene may contribute to the susceptibility for epilepsy in humans. Interestingly, we recently discovered a single-nucleotide polymorphism (SNP), namely the Phe217Leu variation (rs1047552) located in exon 6 of the human *APH1B* gene, that in transfection studies affected γ-secretase cleavage activity, suggesting a functional relevance for this SNP (van Loo et al., 2008). In the present study, we therefore tested in Caucasian case-control cohorts for association of the functional Phe217Leu genetic variation in *APH1B* with epilepsy.

Materials and Methods

Subjects

The Dutch cohort consisted of 175 Caucasian IGE patients and 277 Caucasian blood bank controls from the same geographic region. The study was approved by the Medical Ethical Committee of the University Medical Center Utrecht and all participating patients provided informed consent. The patient group from the United Kingdom (Kent) consisted of 226 Caucasian patients with a broad IGE phenotype. Patients with a diagnosis of IGE and generalized spike/wave on EEG were recruited through East Kent Hospitals. Control DNA was obtained from 309 unrelated individuals with the same ethnic background and recruited from the same region, group-matched for age and sex, and no history of epilepsy or blackouts. Ethical approval was obtained. The second patient group from the United Kingdom (Glasgow) consisted of 595 Caucasian patients recruited from out-patient clinics at the Epilepsy Unit, Western Infirmary, Glasgow, Scotland. DNA sample collection and analysis was approved by the West Research Ethics Committee, North Glasgow University Hospitals NHS Trust and all participants provided written, informed consent. The epilepsy was classified as localization-related in 422 (71%) patients, idiopathic generalised in 157 (26%), and was unclassified in the remaining 16 (3%) individuals.

APH1B genotyping

Following DNA isolation according to standard procedures, *APH1B* Phe217Leu genotyping was performed using the invader assay (Prevention Genetics) or via allele-specific PCR using primers specific for single-nucleotide polymorphism (SNP) rs1047552. For the latter approach, the PCR mixture contained ~20 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.25% Nonidet P-40, 0.25% Tween-20, 200 μM each of the four deoxynucleotide triphosphates, 0.8 units of *Taq* polymerase (MBI Fermentas) and 0.6 μM of each of four oligonucleotide primers (outer/general primers: forward: 5'-TGCCTTCTAGGGTTACCATCTGA-3' and reverse: 5'-AGTCGGCTTTACACTGTCCCA-3'. Inner/specific primers: forward specific for the "T-allele": 5'-AATAAACCTGGCGTCAGCATTT-3' and reverse specific for the "G-allele": 5'-GCCCATGAGCACCAGGATTATC-3') in a total volume of 20 μl. Reactions were amplified for 30 cycles at 94°C for 1 min, 68°C for 30 sec and 72°C for 1 min, followed by a final extension step at 72°C for 10 minutes. PCR products were analysed on 1% agarose gels. The homozygous TT- and GG-genotypes gave two bands, (621 / 370 and 621 / 294 base pairs, respectively), whereas the heterozygous TG-genotype resulted in three bands (621, 370 and 294 base pairs).

Statistical analysis

Differences between cases and controls were analysed by standard contingency table analysis using two-tailed χ^2 test probabilities. Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE). The strength of association was summarized by using the odds ratio (OR with 95% confidence intervals (CI)), in which an OR greater than 1.0 indicated a positive association (GraphPad Software Inc, San Diego, CA, USA).

Results

In search for potential association of the functional *APH1B* Phe217Leu genetic variation with epilepsy, we first tested a Dutch cohort consisting of 175 IGE patients and 277 controls from the same geographic region. Interestingly, allele frequencies were significantly different between the Dutch epilepsy patients and the control population ($\chi^2 = 3.90$, $df = 1$, $p = 0.048$; OR = 2.42; CI = 0.98-5.99), with a higher prevalence of the risk allele (G-allele) in the patients (table 1).

For association studies of disorders with a high level of heterogeneity and in particular when a low minor allele frequency (MAF) of the susceptibility polymorphism is involved (as holds for the Phe217Leu polymorphism), reliable association data are difficult to obtain. Consequently, additional populations are necessary to increase the power in order to allow a statistically significant disorder/genotype relationship. We therefore decided to screen a second cohort, consisting of 226 Caucasian patients with IGE and 309 Caucasian controls (cohort from Kent). This second cohort also showed a higher prevalence of the risk allele in the cases, albeit not statistically different from the control population ($\chi^2 = 0.21$, $df = 1$, $p = 0.65$; OR = 1.19; CI = 0.56-2.53) (table 1). A third group consisted of 595 Caucasian epilepsy patients from Glasgow (including 157 patients with the IGE subtype). Analysis of these patients showed a higher prevalence of the risk allele in the IGE subtype (MAF of 4.5%) compared to the non-IGE subtype (MAF of 2.9%) (table 1), indicating an enrichment of the *APH1B* Phe217Leu risk allele in the IGE subtype. All genotype distributions tested (cases and controls) did not significantly deviate from HWE (data not shown).

Discussion

In the present study on three independent Caucasian groups, we show that the *APH1B* Phe217Leu polymorphism may be a risk factor for epilepsy, more specifically for the IGE subtype. Although the cohort from the United Kingdom failed to reach significant levels, a higher prevalence of the risk allele was found among IGE patients from both the Dutch and the United Kingdom cohort when compared with

Table 1. Genotype and allele frequencies for the *APH1B* Phe217Leu variation in epilepsy case-control studies

		Genotype Frequencies (%)			Allele Frequencies (%)			
	n	TT	TG	GG	T	G	*p*	OR
The Netherlands								
Patients (IGE)	175	93.1	6.9	0	96.6	3.4	0.048*	2.42
Controls	277	97.1	2.9	0	98.6	1.4		
United Kingdom (Kent)								
Patients (IGE)	226	94.2	5.8	0	97.1	2.9	0.65	1.19
Controls	309	95.1	4.9	0	97.6	2.4		
United Kingdom (Glasgow)								
Patients (IGE)	157	91.1	8.9	0	95.5	4.5	0.17	1.59
Patients (Non-IGE)	438	94.5	5.3	0.2	97.1	2.9		

*Standard Chi-square tests were applied to evaluate the association of the APH1B Phe217Leu variation with epilepsy. OR = odds ratio; *p<0.05.*

healthy controls. Furthermore, analysis of a third group of epilepsy patients (United Kingdom, Glasgow), showed a higher prevalence of the risk allele in the IGE patients compared to the non-IGE patients. Thus, our data suggest that the *APH1B* Phe217Leu polymorphism may be a risk factor for IGE. Nevertheless, replication of our results in other IGE cohorts and extrapolation to other epilepsy subtypes will be essential to reveal whether the *APH1B* Phe217Leu SNP constitutes a risk factor solely for IGE or for a wide variety of epilepsy subtypes.

A functional defect in the *APH1B* gene was also observed in the APO-SUS/-UNSUS rat model (Coolen et al., 2005), an animal model with neurodevelopmental alterations (Ellenbroek et al., 1995; Ellenbroek and Cools, 2002), including increased bursts of spike wave discharges (de Bruin et al., 2000). The gene-dosage imbalance of the *Aph-1b* gene (three gene copies in APO-UNSUS rats and one or two gene copies in APO-SUS rats) segregated with differences in γ-secretase cleavage activity and a number of phenotypic characteristics (Coolen et al., 2005). Furthermore, we recently found a reduced γ-secretase cleavage activity in *Aph-1abc*$^{-/-}$ mouse embryonic fibroblasts (MEFs) transfected with the APH1B Leu217 variant when compared with *Aph-1abc*$^{-/-}$ MEFs transfected with the Phe217 variant, indicating that the *APH1B* Phe217Leu polymorphism represents a functional polymorphism (van Loo et al., 2008). We therefore speculate that a subtle effect on *APH1B* expression or function (copy number variation in rat or Phe217Leu variation in human, respectively) may affect γ-secretase signalling and consequently influence neuronal functioning, including

the interplay of excitatory and inhibitory neurons that can generate the abnormal oscillations as seen in people with epilepsy.
Although the *APH1B* Phe217Leu polymorphism has been previously associated with Alzheimer's disease and premature coronary atherosclerosis (Poli et al., 2008; van Loo et al., 2008), it has not been considered before in the context of epilepsy. Interestingly, a number of γ-secretase substrates, namely Scnb2, GluR3 and HLA-A2, have been found to be involved in the development of epileptic seizures. Scnb2 homozygous knock-out mice (*Scn2b*$^{-/-}$) showed a deficient regulation of electrical excitability in the brain, resulting in an increased susceptibility to epileptic seizures (Chen et al., 2002). Immunization of rabbits with the GluR3 protein induced epileptic seizures in the animals and serum samples of people with epilepsy contained more often antibodies to GluR3 compared to healthy controls (Rogers et al., 1994; Wiendl et al., 2001; Mantegazza et al., 2002), indicating a link between GluR3 and the development of epileptic seizures. For the γ-secretase substrate HLA-A2, a higher percentage of antigen was found in the sera of epilepsy patients compared to controls (Fontana et al., 1978). Since we now find that the human γ-secretase polymorphism Phe217Leu may be associated with epilepsy and rats with a reduced number of *Aph1b* gene copies display an increased incidence of bursts of bilateral synchronous spike wave discharges (de Bruin et al., 2000), we hypothesize that in a subset of individuals a disturbed γ-secretase signalling may lead to epileptic alterations.
Altogether, our results suggest that γ-secretase signalling may be a critical process for the generation of normal brain rhythms and may represent a candidate pathway for IGE.

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Chapter B3

Susceptibility for HIV-1 infection and the γ-secretase pathway

With
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Submitted

Abstract

Susceptibility for human immunodeficiency virus type 1 (HIV-1) infection is individual specific and may be influenced by host genetics. Based on recent findings with a rat model, we speculated that the γ-secretase pathway may be associated with an individuals' infection susceptibility. We here find that in a Xhosa indigenous South African cohort a functional single-nucleotide polymorphism in the γ-secretase component APH1B shows a tendency for association with HIV-1 infection ($p=0.087$). Interestingly, the polymorphism was significantly associated in a second, Caucasian cohort for HIV-1 infection ($p=0.049$). Together, our results suggest a role for the γ-secretase pathway in susceptibility for HIV-1 infection.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection is the causative factor of acquired immunodeficiency syndrome (AIDS) and has a strikingly increasing prevalence rate, especially in developing countries (McCutchan, 2006). The susceptibility to HIV-1 infection is remarkably dependent on the individual; whereas some HIV-1 infected individuals show a rapid progression to AIDS, others exhibit an asymptomatic survival despite their HIV-1 infection (Klein and Miedema, 1995). In addition, some individuals have remained HIV-1 negative despite reported exposure to HIV-1 during high-risk sexual behaviour. Host factors, such as genetic mutations and single-nucleotide polymorphisms (SNPs), may therefore contribute to HIV-1 infection susceptibility and disease progression. A number of genetic variants, including variations within the chemokine receptor 5 (*CCR5*) gene, have been found to be associated with the acquisition of HIV-1 infection and its clinical course (Reiche et al., 2007). However, the key genes and pathways involved in HIV-1 infection susceptibility are not clear.

Apomorphine-susceptible (APO-SUS) and apomorphine-unsusceptible (APO-UNSUS) Wistar rats have been shown to differ in immunological parameters (Ellenbroek and Cools, 2002). For instance, APO-SUS rats displayed a stronger T helper 2 (Th2)-dependent IgE response upon worm infection, a lower Th1-mediated autoimmune encephalomyelitis response and a disturbed Th1/Th2 balance when compared to APO-UNSUS rats (Kavelaars et al., 1997). Thus, the rats appear to differ in host resistance or susceptibility to inflammation and infection. We recently identified a gene-dosage imbalance of the γ-secretase component Aph-1b in the APO-SUS/-UNSUS rat model (Coolen et al., 2005). The γ-secretase enzyme complex cleaves a variety of type I transmembrane proteins, including Notch1-4, β-amyloid precursor protein, sialophorin (CD43), HLA-A2 and syndecan-3 (Andersson et al., 2005; Parks and Curtis, 2007).

Intrigued by the findings with the rat model, we wondered whether an association between *APH1B* and susceptibility to HIV-1 infection would exist and thus whether γ-secretase signalling should be considered as a new susceptibility pathway for HIV-1 infection. Intriguingly, we recently discovered a SNP (rs1047552, T>G, Phe217Leu) in exon 6 of the human *APH1B* gene (located on chromosome 15q22.2 and spanning 28.3 kb; MIM# 607630, also known as presenilin stabilization factor-like, *PSFL*) that was found to be functional in transfection studies on Phe217Leu-mutated APH1B (van Loo et al., 2008). In the present study, we therefore tested for association of this non-synonymous SNP with HIV-1 infection in both an African and a Caucasian case-control study.

Materials and Methods

Subjects

The South African study representing unrelated Africans of Xhosa ethnic descent and residing in the Western Cape of South Africa has been described previously (Hayes et al., 2002). We analysed a total of 198 HIV-1-seropositive (61 males and 137 females) and 112 HIV-1-seronegative individuals (42 males and 70 females). The Ethics Review Committee of the University of Stellenbosch approved the study protocol (#98/158). The Dutch cohort consisted of 1210 Caucasian male subjects: 335 HIV-1-seropositive individuals recruited via the Amsterdam Cohort on HIV-1 infection and AIDS between October 1984 and March 1986 (de Roda Husman et al., 1997) and 875 randomly selected Dutch controls from the Sanquin Common Control Collection (SANQUIN-CCC): a Dutch national repository of common controls set up in 2007 as part of the Bloodomics project by Sanquin. Volunteers were recruited at their blood donation session at one of the collection sites of the Sanquin Blood Bank covering the north west of the Netherlands. Therefore more than >95% of these donors lived in the Dutch postal code area 1000-4000. This area was chosen while the Amsterdam cohort of HIV-1 individuals geographically overlaps the region of the blood donor cohort serving as control donors. Informed consent was obtained from all study participants.

APH1B genotyping

Following the isolation of genomic DNA according to standard procedures, *APH1B* Phe217Leu genotyping was performed via denaturing gradient gel electrophoresis (DGGE) of PCR fragments, a Taqman assay (Applied Biosystems, Foster City, CA, USA) or allele-specific PCR using primers specific for SNP rs1047552 (primers and conditions available on request). The presence of the SNP was confirmed by Sanger sequencing.

Statistical analysis

Genotype frequencies were tested for the Hardy-Weinberg equilibrium. Differences between cases and controls were analysed using two-tailed χ^2 probabilities, and 95% confidence intervals (95% CIs) were calculated for the odds ratios (ORs). A p-value < 0.05 was considered statistically significant (GraphPad Software Inc, San Diego, CA, USA).

Results

In search for potential association of the non-synonymous Phe217Leu polymorphism

(rs1047552) of human *APH1B* with HIV-1 infection, we tested a case-control cohort of individuals belonging to the Xhosa ethnic group of South Africa (198 HIV-1-seropositive patients and 112 non-infected individuals). Interestingly, we found a trend towards an association (χ^2 = 2,94, *df* = 1, p =0.087; odds ratio [OR] for G-allele = 2.33; CI = 0.86-6.30) (table 1). Subdivision of the sample based on gender showed a slightly higher prevalence of the minor allele variant in the males compared to females (OR in males: 2.88; OR in females: 2.09). Since the size of the African cohort was restricted and we are dealing here with a relatively low frequency of the risk allele (2.2% in the African controls), making reliable association difficult to establish, we decided to extend our analysis by testing a second case-control cohort. Our second study comprised 1210 Dutch males, consisting of 335 HIV-1-seropositive patients and 875 non-infected controls. Intriguingly, a significant association of SNP Phe217Leu was found in this male cohort with an OR of 1.67 (χ^2 = 3.86, *df* = 1, p =0.049; CI = 1.00-2.81) (table 1). All genotype distributions tested (cases and controls) fulfilled the Hardy-Weinberg criteria.

Table 1. Genotype and allele frequencies for the *APH1B* Phe217Leu variation in two case-control studies on susceptibility for HIV-1 infection

		Genotype Frequencies (%)			Allele Frequencies (%)			
	n	TT	TG	GG	T	G	*p*	OR
South Africa								
HIV-	112	95.5	4.5	0	97.8	2.2		
HIV+	198	90.4	9.1	0.5	94.9	5.1	0.087	2.33
The Netherlands								
HIV-	875	95.7	4.3	0	97.8	2.2		
HIV+	335	92.8	7.2	0	96.4	3.6	0.049*	1.67

*Standard Chi-square tests were applied to evaluate the association with HIV-1 infection. OR = odds ratio; *p<0.05.*

Discussion

In this study, we found a higher prevalence of the *APH1B* Phe217Leu risk allele among HIV-1-infected individuals. The increase of the risk allele in the Xhosa ethnic group from South Africa barely failed to reach significant levels, probably due to the low number of individuals tested, whereas the increase of the risk allele as observed in the larger Caucasian study represented a statistically significant association. Since the first transmission from simian primates to humans (Gao et al., 1999), HIV-1 and HIV-2 have differentiated into several subtypes (McCutchan, 2006). These subtypes

can be related to geographic areas, with HIV-1 subtype B predominating in Western Europe and HIV-1 subtype C in South Africa. Together with viral diversity, host genetic diversity has also proven to be an important factor contributing to HIV-1 susceptibility. In this respect, one of the most relevant observations was the absence in the Xhosa population of the common Caucasian *CCR5* 32-base pair deletion mutation, known to be generally protective against HIV-1 infection and disease progression (Petersen et al., 2001). We thus conclude from our findings that the *APH1B* Phe217Leu polymorphism may be a susceptibility factor for HIV-1 infection in both Caucasian and African populations, and under the influence of different subtypes of HIV-1 infection (subtype B and subtype C infection, respectively).

γ-Secretase and *APH1B* have not been considered before in relation to HIV-1 infection. Interestingly, at least three γ-secretase substrates may be linked to the mechanism involved in HIV-1 infection, namely the substrates HLA-A2, CD43 and syndecan-3. HLA-A2 is a major histocompatibility complex class I protein involved in immune control of HIV-1 infection (reviewed by Carlson and Brumme, 2008), CD43 may lower the threshold necessary for HIV-1 viral replication and as such modulate HIV-1 progression (Barat and Tremblay, 2002), and syndecans may act as *in trans* HIV-1 receptors to allow HIV-1 pathogens to enter into host cells (Bobardt et al., 2003). Detailed studies are however necessary to unravel the pathological process in HIV-1-seropositive individuals carrying the *APH1B* Phe217Leu polymorphism. In this connection, it is important to note that transfection of the Leu217 variant of the APH1B protein into *Aph-1abc*$^{-/-}$ mouse embryonic fibroblasts resulted in a reduced γ-secretase cleavage activity towards syndecan-3, indicating that the *APH1B* Phe217Leu polymorphism represents a functional SNP (van Loo et al., 2008).

In conclusion, our results show that the Phe217Leu polymorphism in the *APH1B* gene has a higher prevalence among HIV-1 infected individuals, linking the γ-secretase pathway to susceptibility for HIV-1 infection.

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Chapter B4

A γ-secretase polymorphism and complex disorders

With
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Abstract

A hallmark of the pathogenesis of Alzheimer's disease (AD) is an affected γ-secretase pathway. The γ-secretase enzyme cleaves multiple transmembrane proteins involved in a variety of developmental processes. We have recently found that a functional non-synonymous single-nucleotide polymorphism (SNP) in the γ-secretase component APH1B (Phe217Leu; rs1047552) is associated not only with AD but also with atherosclerosis, epilepsy and HIV-1 infection. We now find that this SNP associates with colorectal cancer as well. In addition, a higher prevalence of the risk allele was observed in patients with bipolar disorder, autism, attention deficit hyperactivity disorder, dyslexia, rheumatoid arthritis, celiac disease, throat cancer, prostate cancer and lung cancer. However, probably due to the low numbers of cases and the low frequency of the risk allele, these associations proved not to be statistically different from the respective controls. No higher prevalence of the risk allele was found for schizophrenia and depression. Based on our study, involving 7,062 patients and 5,872 controls, we conclude that the γ-secretase pathway contributes not only to AD, but may also play a role in the pathogenesis of a number of other complex disorders.

Introduction

The γ-secretase enzyme complex is best known for its involvement in the sequential cleavage of amyloid-β precursor protein (APP) in the neurodegenerative disorder Alzheimer's disease (AD). The complex consists of four different integral membrane proteins, namely presenilin (either presenilin 1 or presenilin 2), nicastrin, presenilin-enhancer 2 and Aph1 (Fortini, 2002; Francis et al., 2002; Goutte et al., 2002; Kimberly and Wolfe, 2003). The enzyme is responsible for the proteolytic processing of a wide variety of type I transmembrane proteins, including APP and its relatives the APP-like proteins APLP1 and APLP2, Notch1-4, neuregulin, N- and E-cadherin, and ErbB4 (reviewed by Parks and Curtis, 2007).
Recently, we observed a disturbed γ-secretase cleavage activity in a rat model with a complex phenotype (Coolen et al., 2005). The disturbance was caused by a gene-dosage imbalance of the gene encoding the γ-secretase component Aph-1b. Whereas apomorphine unsusceptible (APO-UNSUS) rats harbour three copies of the *Aph-1b* gene, their phenotypic counterparts (APO-SUS rats) have only one or two gene copies. Interestingly, the number of gene copies was associated with a number of phenotypic APO-SUS and -UNSUS characteristics (Coolen et al., 2005).
Since the APO-SUS and -UNSUS rats display a complex phenotype (reviewed by Ellenbroek and Cools, 2002), affected γ-secretase activity may modulate a number of phenotypes and contribute to the pathogenesis of disorders with a complex aetiology. Association of a functional non-synonymous single-nucleotide polymorphism (SNP) in the human *APH1B* gene (rs1047552; Phe217Leu) has previously been found with AD (Poli et al., 2008), atherosclerosis (van Loo et al., 2008), epilepsy and HIV-1 infection (chapters B2-B3). In the present study, we wondered whether the *APH1B* polymorphism may also contribute to the susceptibility of other complex disorders. Therefore, we searched in Caucasian case-control cohorts for association of the *APH1B* Phe217Leu variation with a number of disorders with a complex aetiology.

Materials and methods

Subjects

Schizophrenia

Norway. The Norwegian study represented 324 Caucasian individuals and has been described previously (Birkenaes et al., 2007). Briefly, patients were recruited from the Oslo TOP (Thematic Organized Psychoses Research) Study, an ongoing study from the University and University Hospital of Oslo (Norway), and were selected according the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). Two senior clinicians continuously trained and supervised a group

of research fellows in order to secure the quality of the clinical assessments. The majority (90%) of the patients were ethnical Norwegian, i.e. the patient and both parents were born in Norway, while in a minor fraction of the cases (10%) one parent was born outside Norway in another North-western European country. Controls (n = 192) were randomly selected from statistical records of persons from the same catchments area as the patient groups. Only subjects born in Norway were contacted by letter and invited to participate. All controls were of Caucasian origin; about 85 % had two Norwegian parents, the rest one parent from other European origin. Moreover, all participants had to have Norwegian as their first language or have received their compulsory schooling in Norway. The control subjects were screened by clinical interviews and with the Primary Care Evaluation of Mental Disorders (PRIME-MD). None of the control subjects had a history of moderate/severe head injury, neurological disorder, mental retardation or an age outside the range of 18-60 years. Healthy subjects were excluded if they or any of their close relatives had a lifetime history of a severe psychiatric disorder (schizophrenia, bipolar disorder and major depression), a history of medical problems thought to interfere with brain function (hypothyroidism, uncontrolled hypertension and diabetes), or significant illicit drug use. All subjects had given written informed consent of participation and the study has been approved by The Norwegian Scientific-Ethical Committees and the Norwegian Data Protection Agency.

Sweden. The first Swedish cohort for schizophrenia (denoted as Sweden1) consisted of 220 schizophrenic patients and 281 non-psychiatric controls (all Caucasian individuals) selected from the northwestern part of Stockholm County (Gustavsson et al., 1999). Patients were recruited according to DSM-III-R. Control subjects were either re-examined healthy individuals, mainly staff and medical students, or subjects drawn from the general population for biological psychiatric studies performed at the Karolinska Institute. The study was approved by the Ethics Committee of the Karolinska Hospital. All subjects participated after giving informed consent. The second Swedish cohort for schizophrenia (Sweden2) was comprised of 450 unrelated SZ patients from the Northern part of Sweden (mean age at inclusion 53.1 ± 15.1). The individuals were thereafter examined at multiple time points, a majority up to the year 2005, by research psychiatrists and nurses. Relevant parts of semi-structured interview instrument i.e. the Mini-International Neuropsychiatric Interview-MINI (Sheehan et al., 1998), the Diagnostic Interview for Genetic Studies-DIGS (http://zork.wustl.edu/nimh/home/m_DIGS2.0_Interview.html), the Family Interview for Genetic Studies-FIGS (http://zork.wustl.edu/nimh/home/FIGSInterview.html) and the Schedules for Clinical Assessment in Neuropsychiatry-SCAN (Wing et al., 1990) were used when deemed necessary. The diagnoses were made according to the DSM-IV criteria. The mean age of disease-onset for the patients was 24.8 ±

7.3. The control individuals were randomly selected from the Betula project, a large population-based prospective study that is described in detail elsewhere (Nilsson et al., 1997). The random unrelated control population (n = 450; mean age at inclusion = 58.0 ± 13.0) was recruited from the same geographical region of Northern Sweden as the patients. All control individuals and SZ patients were Caucasians and their mother tongue was Swedish. Participants were only included in the study after signing an informed consent, and the study was approved by the Medical Ethical Committees of the universities of Umeå and Antwerp. The association sample was controlled for population stratification by genotyping 37 microsatellite (STR) markers using standard genotyping and scoring methods. Statistical tests for population stratification were performed with the program STRUCTURE (http://pritch.bsd.uchicago.edu/structure.html). No population substructure was observed in the association sample (data not shown).

Denmark. The Danish cohort consisted of 407 Caucasian schizophrenic patients and 811 unrelated Caucasian controls. Patients were recruited to the Danish Psychiatric Biobank from the psychiatric departments at the six hospitals in the Copenhagen region, and were clinically diagnosed with schizophrenia or schizoaffective disorder according to ICD-10. An experienced research- and consultant psychiatrist verified high reliability of the clinical diagnoses using OPCRIT semi-structured interviews. The mean duration of hospitalization was 12.8 (± 8.6) years. The majority (87%) of the patients were ethnical Danish, i.e. the patients and both parents were born in Denmark, while in a minor fraction of the cases (13%) one parent was born outside Denmark in another North-western European country. The healthy control subjects were recruited among 15.000 blood-donors from the Danish Blood Donor Corps in the Copenhagen area. The Donor corps includes >5% of the Danish population who donate blood on a voluntary and unpaid basis. Apparent behavioural abnormality was an exclusion criterion and all individuals stated that they felt completely healthy with a possibility to discuss any health-related issues with a physician. Two unrelated healthy control subjects of Danish Caucasian origin were matched to each patient on gender, year of birth and month of birth. The study was approved by the Danish Scientific-Ethical Committees and the Danish Protection Agency. All patients had given written informed consent before inclusion into the project.

The Netherlands. The Dutch group consisted of 335 schizophrenic patients recruited through mental health services and relatives' support groups in the Netherlands (selected according DSM-IV) and 314 non-psychiatric Dutch controls.

Belgium. The Belgium sample consisted of 515 schizophrenic patients and 246 randomly selected controls from the same geographic region (Leuven, Belgium). Patients were selected according the DSM-IV criteria (schizophrenia: disorganized type (295.1): 10 patients; paranoid type (295.3): 213 patients; schizophreniform

disorder (295.4): 58 patients; residual type (295.6): 2 patients; schizoaffective disorder (295.7): 106 patients; undifferentiated type (295.9): 126 patients).
USA. A total of 793 Caucasian individuals were derived from the PrecisionMed Sample Bank collection (San Diego, California, USA). Controls (n=300) had no (family) history of neuropsychiatric disease and were recruited from the same geographic region as the patients with schizophrenia (n=493). All subjects had given written informed consent of participation.

Bipolar disorder
The Norwegian cohort consisted of 116 Caucasian patients with bipolar disorder (recruited from the Oslo TOP Study described above) and 192 non-psychiatric Caucasian controls selected from statistical records of persons from the same area as the patient groups (see above). The Belgium cohort consisted of 60 Caucasian patients and 246 selected Caucasian controls from the same geographic region (Leuven, Belgium). Informed consent was obtained from all individuals.

Autism
The Belgium cohort consisted of 98 patients with autism and 246 randomly selected controls from the same geographic region (Leuven, Belgium).

Attention deficit hyperactivity disorder (ADHD) and dyslexia
The Dutch neurodevelopmental group (ADHD and dyslexia) consisted of 84 patients with ADHD, 91 patients with dyslexia and 314 non-psychiatric Dutch controls.

Depression
For the depression cohort, 300 patients with depression and 300 controls with no (family) history of neuropsychiatric disease and recruited from the same geographic region as the patients were derived from the PrecisionMed Sample Bank collection (San Diego, California, USA). All subjects had given written informed consent of participation.

Rheumatoid arthritis
The Swedish cohort for rheumatoid arthritis consisted of 393 Caucasian sporadic rheumatoid arthritis patients and 424 Caucasian controls from the same geographical region (Umeå, Northern part of Sweden), matched for age, sex and area of residence. Informed consent of participation was obtained from all individuals.

Celiac disease
The Dutch case-control cohort for celiac disease consisted of 404 independent

celiac disease patients and 277 independent controls, all of Dutch Caucasian origin. Patients were diagnosed according to the ESPGHAN criteria and only patients with a biopsy-proven Marsh III lesion were included (Van Belzen et al., 2003). The study was approved by the Medical Ethics Committee of the University Medical Centre in Utrecht and informed consent was obtained from all individuals.

Cancer

The first Dutch cancer group (Nijmegen) consisted of 362 colorectal cancer (CRC) patients, 284 throat cancer patients and 591 Dutch controls. All subjects studied were Caucasians of Dutch origin. Cases were recruited at the University Medical Centre St Radboud (Nijmegen, The Netherlands) and were classified according to the location of the tumour. CRC patients have been described in more detail elsewhere (van der Logt et al., 2006). The control subjects were from the Dutch blood bank (Utrecht and Amsterdam). The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. The second Dutch cancer group (Maastricht) consisted of 700 CRC patients and 1772 matched controls from The Netherlands Cohort Study on diet and cancer (NLCS), which has been described in detail elsewhere (van den Brandt et al., 1990; Brink et al., 2004).

Subjects for the prostate cancer study were participants in the Melbourne and Perth arms of the Risk Factors for Prostate Cancer (RFPC) study, an Australian population-based case-control study of prostate cancer conducted between 1994 and 1998 and described in detail elsewhere (Giles et al., 2001; Severi et al., 2003). 820 Patients with prostate cancer (histopathologically-confirmed adenocarcinoma of the prostate diagnosed before age 70 years) and 733 healthy controls (randomly selected from males on the State Electoral Rolls and matched to the age distribution of the prostate cancer cases) were included.

For the lung cancer group, 798 patients with non-small cell lung carcinoma (NSCLC) were selected from patients treated at The Prince Charles Hospital (Brisbane, Australia) from 1980 to 2003 (Larsen et al., 2005, 2006). Controls consisted of healthy female smokers (n = 72) attending a smoking cessation clinic held at the hospital from 2000 to 2003 and male controls from the prostate study described above (n = 733).

APH1B genotyping

Following the isolation of genomic DNA, *APH1B* Phe217Leu genotyping was performed via Taqman assays (Applied Biosystems, Foster City, CA, USA), denaturing gradient gel electrophoresis (DGGE) of PCR fragments or allele-specific PCR using primers specific for SNP rs1047552 (outer/general primers: forward: 5'-TGCCTTCTAGGGTTACCATCTGA-3' and reverse:

5'-AGTCGGCTTTACACTGTCCCA-3'. Inner/specific primers: forward specific for the "T-allele": 5'-AATAAACCTGGCGTCAGCATTT-3' and reverse specific for the "G-allele": 5'-GCCCATGAGCACCAGGATTATC-3').

Statistical analysis

Genotype frequencies were tested for the Hardy-Weinberg equilibrium. Standard contingency table analysis using two-tailed χ^2 test probabilities were used to compare allele frequencies between cases and controls. Odds ratios (95% confidence intervals (CI)) were calculated as an index of the association of the *APH1B* genotypes with the diseases (GraphPad Software Inc, San Diego, CA, USA). Heterogeneity between schizophrenia cohorts was assessed using Cochran's Q statistic, and meta-analysis was performed using fixed-effects models (EasyMa2001). A p-value < 0.05 was considered statistically significant.

Results

APH1B Phe217Leu polymorphism in schizophrenia

To examine a possible association between the *APH1B* Phe217Leu polymorphism and disorders with a complex aetiology, we first tested seven cohorts for susceptibility to schizophrenia (cohorts from Norway, Sweden (2 cohorts), Denmark, the Netherlands, Belgium and California, USA). The total sample comprised 2,552 individuals affected with schizophrenia and 2,594 controls (for a detailed description of the samples, see Materials and Methods). Despite a positive tendency in the initial screening of a small cohort in the early stages of the project, analysis of the seven case-control groups showed no statistically significant difference in any of the cohorts tested (table 1 and figure 1). In addition, a meta-analysis of the seven cohorts or simply pooling revealed no excess of the risk allele (meta-analysis: $\chi^2 = 0.025$, $df = 1$, p = 0.87; common relative risk = 0.98; CI = 0.77-1.25; pooling: $\chi^2 = 0.03$, $df = 1$, p = 0.86; odds ratio (OR) = 0.98; CI = 0.77-1.25).

APH1B Phe217Leu polymorphism in bipolar disorder

We then studied whether the Phe217Leu variation associates with bipolar disorder. First, we tested a bipolar disorder cohort from Norway (116 Caucasian patients with bipolar disorder and 192 Caucasian controls). The minor allele (G-allele) was more frequent in the bipolar disorder patients than in the control population, albeit not statistically different ($\chi^2 = 0.55$, $df = 1$, p = 0.55; OR for G-allele = 1.34; CI = 0.52-3.43) (table 2). Our second sample comprised a Belgium case-control cohort consisting of 60 patients with bipolar disorder and 246 controls. Interestingly, allele frequencies were significantly different between the control population and

Table 1. Genotype and allele frequencies for *APH1B* Phe217Leu polymorphism in schizophrenia case-control studies

		Genotype Frequencies (%)			Allele Frequencies (%)				
	n	TT	TG	GG	T	G	HWE	*p*	OR
Norway									
Patients	132	93.9	5.3	0.8	96.6	3.4	0.025^	0.55	1.32
Controls	192	94.8	5.2	0	97.4	2.6	0.71		
Sweden1									
Patients	220	91.8	8.2	0	95.9	4.1	0.53	0.66	1.16
Controls	281	92.9	7.1	0	96.4	3.6	0.54		
Sweden2									
Patients	450	94.9	4.9	0.2	97.3	2.7	0.22	0.88	1.05
Controls	450	95.1	4.7	0.2	97.4	2.6	0.18		
Denmark									
Patients	407	95.8	4.2	0	97.9	2.1	0.67	0.45	0.80
Controls	811	94.9	4.9	0.1	97.4	2.6	0.53		
The Netherlands									
Patients	335	97.0	2.7	0.3	98.4	1.6	0.0021^	0.76	1.15
Controls	314	97.1	2.9	0	98.6	1.4	0.80		
Belgium									
Patients	515	96.5	3.5	0	98.3	1.7	0.69	0.36	0.71
Controls	246	95.1	4.9	0	97.6	2.4	0.69		
USA									
Patients	493	93.1	6.5	0.4	96.3	3.7	0.086	0.99	1.00
Controls	300	92.6	7.3	0	96.3	3.7	0.51		
Pooled									
Patients	2552	95.0	4.8	0.2	97.4	2.6	0.011^	0.86	0.98
Controls	2594	94.8	5.2	0.08	97.3	2.7	0.90		

Standard Chi-square tests were applied to evaluate the association with schizophrenia. HWE = Hardy-Weinberg equilibrium; OR = odds ratio; ^ = deviation from Hardy-Weinberg ($p<0.05$).

the bipolar disorder patients ($\chi^2 = 5.45$, *df* = 1, p = 0.041; OR = 2.86; CI = 1.14-7.16), with a higher prevalence of the risk allele in the patients, as was seen in the Norwegian cohort. Pooling the Norwegian and Belgium samples showed a tendency for association ($\chi^2 = 3.46$, *df* = 1, p = 0.063; OR = 1.85; CI = 0.96-3.56) (table 2).

APH1B Phe217Leu polymorphism in autism, ADHD, dyslexia and depression

Next, we performed preliminary association studies on four other complex disorders,

namely autism, ADHD, dyslexia and depression. For each disorder, one cohort was tested. Three of the four cohorts showed a higher prevalence of the risk allele in the patient populations (autism: OR = 2.15; ADHD: OR = 1.68; dyslexia: OR = 1.55), whereas the depression cohort displayed a lower prevalence of the risk allele in the patients (OR = 0.86). However, the cohorts consisted of only a limited number of patients and the associations were not statistically different (table 2).

Table 2. Genotype and allele frequencies for *APH1B* Phe217Leu polymorphism in bipolar disorder, autism, ADHD, dyslexia and depression case-control studies

		Genotype Frequencies (%)			Allele Frequencies (%)				
	n	TT	TG	GG	T	G	HWE	*p*	OR
Bipolar disorder									
Norway									
Patients	116	93.1	6.9	0	96.6	3.4	0.70	0.55	1.34
Controls	192	94.8	5.2	0	97.4	2.6	0.71		
Belgium									
Patients	60	86.7	13.3	0	93.3	6.7	0.58	0.041*	2.86
Controls	246	95.1	4.9	0	97.6	2.4	0.69		
Pooled									
Patients	176	90.9	9.1	0	95.5	4.5	0.53	0.063	1.85
Controls	438	95.0	5.0	0	97.5	2.5	0.59		
Autism (Neth.)									
Patients	98	90.8	8.2	1.0	94.9	5.1	0.12	0.073	2.15
Controls	246	95.1	4.9	0	97.6	2.4	0.69		
ADHD (Neth.)									
Patients	84	95.2	4.8	0	97.6	2.4	0.82	0.50	1.68
Controls	314	97.1	2.9	0	98.6	1.4	0.80		
Dyslexia (Neth.)									
Patients	91	95.6	4.4	0	97.8	2.2	0.83	0.51	1.55
Controls	314	97.1	2.9	0	98.6	1.4	0.80		
Depression (USA)									
Patients	300	93.7	6.3	0	96.8	3.2	0.57	0.63	0.86
Controls	300	92.6	7.3	0	96.3	3.7	0.51		

*Standard Chi-square tests were applied to evaluate the association with bipolar disorder, autism, ADHD, dyslexia and depression. HWE = Hardy-Weinberg equilibrium; OR = odds ratio; * = p<0.05.*

APH1B Phe217Leu polymorphism in rheumatoid arthritis

To examine a possible association between the *APH1B* polymorphism and rheumatoid arthritis, we tested a Swedish case-control cohort consisting of 393 sporadic rheumatoid arthritis patients and 424 matched controls. Interestingly, the risk allele was more frequent in the rheumatoid arthritis patients than in the control population (2.3% and 1.8%, respectively), but not statistically different ($\chi^2 = 0.56$, *df* = 1, p = 0.45; OR = 1.30; CI = 0.65-2.60).

APH1B Phe217Leu polymorphism in celiac disease

We then tested whether the *APH1B* Phe217Leu polymorphism may also contribute to the autoimmune disorder celiac disease. We therefore tested a Dutch case-control cohort consisting of 404 patients with celiac disease and 277 controls. The results showed a higher prevalence of the risk allele in the celiac disease patients, but no significance was reached ($\chi^2 = 0.55$, *df* = 1, p = 0.46; OR = 1.38; CI = 0.59-3.24).

APH1B Phe217Leu polymorphism in cancer

To explore a possible association of the *APH1B* genetic variation with cancer, we first tested a Dutch cohort for CRC, consisting of 362 CRC patients and 591 controls. Interestingly, allele frequencies were significantly different between the Dutch CRC patients and the control population ($\chi^2 = 9.44$, *df* = 1, p = 0.0021; OR = 2.55; CI = 1.38-4.74), with a higher prevalence of the risk allele in the patients (table 3). Next, we decided to screen a second Dutch cohort for CRC. This second cohort also showed a higher frequency of the risk allele, albeit not statistically different ($\chi^2 = 0.83$, *df* = 1, p = 0.36; OR = 1.20; CI = 0.81-1.80). Pooling the CRC samples (1062 Dutch individuals with CRC and 2363 Dutch controls) revealed a significant association ($\chi^2 = 5.99$, *df* = 1, p = 0.014; OR = 1.50; CI = 1.08-2.07) (table 3). Finally, we tested the hypothesis that the genetic variation also contributes to the pathogenesis of other cancers, including throat, prostate and lung cancer. For each of the cancers, one cohort was examined. Interestingly, all three cohorts showed a higher prevalence of the risk allele in the cancer populations (throat cancer: OR = 1.86; prostate cancer: OR = 1.21; lung cancer: OR = 1.13), but the associations proved not to be statistically different (table 3).

Discussion

The tetrameric γ-secretase enzyme complex displays intramembrane proteolysis of more than 30 type I transmembrane proteins that play a role in a large variety of signalling pathways (reviewed by Boulton et al., 2008). Recently, we found substrate-specific alterations in γ-secretase cleavage activity in rats with a complex phenotype,

Table 3. Genotype and allele frequencies for *APH1B* Phe217Leu polymorphism in cancer case-control studies

		Genotype Frequencies (%)			Allele Frequencies (%)				
	n	TT	TG		T	G	HWE	*p*	OR
Colorectal cancer									
Nijmegen (Neth.)									
Patients	362	93.4	6.1	0.6	96.4	3.6	0.020^	0.0021**	2.55
Controls	591	97.1	2.9	0	98.6	1.4	0.72		
Maastricht									
Patients	700	95.1	4.6	0.3	97.4	2.6	0.020^	0.36	1.20
Controls	1772	95.7	4.3	0	97.9	2.1	0.36		
Pooled									
Patients	1062	94.5	5.1	0.4	97.1	2.9	0.0008^	0.014*	1.50
Controls	2363	96.1	3.9	0	98.0	2.0	0.33		
Throat cancer (Neth.)									
Patients	284	94.7	5.3	0	97.4	2.6	0.65	0.079	1.86
Controls	591	97.1	2.9	0	98.6	1.4	0.72		
Prostate cancer (Aus.)									
Patients	820	93.9	6.0	0.1	96.9	3.1	0.81	0.39	1.21
Controls	733	95.2	4.4	0.4	97.4	2.6	0.0002^		
Lung cancer (Aus.)									
Patients	798	94.2	5.6	0.1	97.1	2.9	0.70	0.56	1.13
Controls	805	95.2	4.5	0.4	97.4	2.6	0.0007^		

*Standard Chi-square tests were applied to evaluate the association with cancer. HWE = Hardy-Weinberg equilibrium; OR = odds ratio; * = p<0.05; ** = p<0.01; ^ = deviation from Hardy-Weinberg (p<0.05).*

the so-called APO-SUS rats. The affected γ-secretase cleavage activity was caused by a gene-dosage imbalance of the *Aph-1b* gene (Coolen et al., 2005; Coolen et al., 2006). Furthermore, we found substrate-specific alterations in γ-secretase cleavage activity for a variation in human *APH1B*. Presence of a leucine instead of a phenylalanine at position 217 of the human APH1B protein resulted in a reduced cleavage of syndecan-3, whereas cleavage of APP and N-cadherin were unaffected (van Loo et al., 2008). Interestingly, this polymorphism was associated with AD, epilepsy, atherosclerosis and HIV-1 infection (Poli et al., 2008, van Loo et al., 2008, chapters B2-B3). We now find a higher prevalence of the *APH1B* risk allele in a number of complex disorders (figure 1).

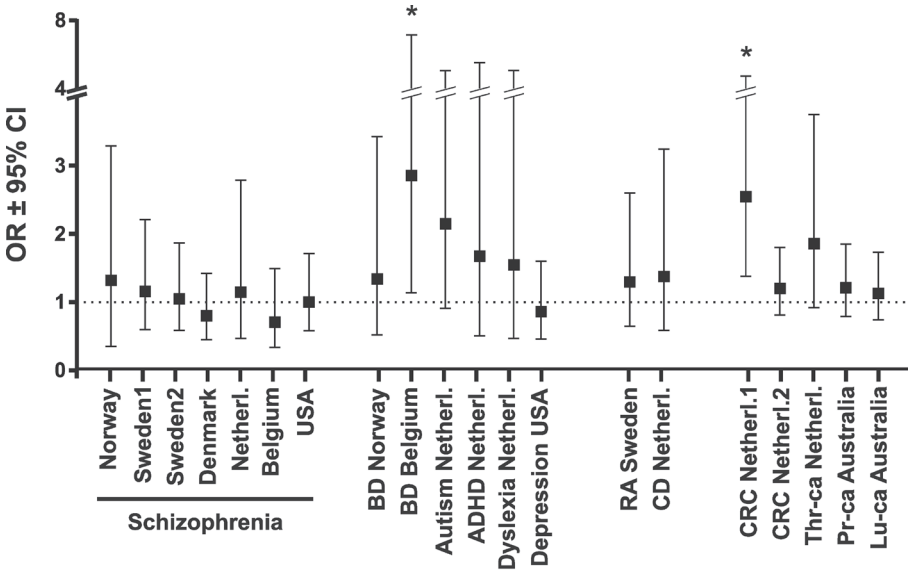

Figure 1. *Odds ratios for the association between the risk allele of the APH1B Phe217Leu polymorphism and a number of complex disorders. Indicated are individual odds ratio (OR) estimates for schizophrenia, bipolar disorder (BD), autism, ADHD, dyslexia, depression, rheumatoid arthritis (RA), celiac disease (CD), colorectal cancer (CRC), throat cancer (Thr-ca), prostate cancer (Pr-ca) and lung cancer (Lu-ca). Vertical lines represent 95% confidence intervals (CIs); *p <0.05.*

Although the presence of the Leu217 allele was enriched in patients with bipolar disorder, autism, ADHD and dyslexia, no higher occurrence of the risk allele was found for schizophrenia and depression, two other neurodevelopmental disorders with a complex aetiology. Yet, the identification of genetic susceptibility factors for neurodevelopmental disorders is complicated, especially when considering the complex interactions of such factors with the environment, the relatively low frequency of the risk allele and heterogeneity within the samples (reviewed by van Loo and Martens, 2007). More extensive analyses based on clinical subtypes and endophenotypically defined patients are necessary for a more definitive conclusion.
Intriguingly, analysis of the four cancer subtypes showed a higher frequency of the Leu217 allele in all patient groups when compared to their healthy controls, but significant association was found only for CRC. At least six of the γ-secretase substrates, namely deleted in colorectal cancer (DCC), Notch, Ephrin-B2, growth hormone receptor (GHR), tyrosinase-related protein-2 (TYRP2) and human leucocyte antigen-A2 (HLA-A2), appear to be involved in the development of intestinal fate and CRC. *DCC* is located on chromosome 18q, a locus affected by allelic losses in almost 70% of all CRC cases (Vogelstein et al., 1988). Furthermore, *DCC* expression was often reduced or even absent in tissues from patients with colorectal cancer

(Fearon et al., 1990; Itoh et al., 1993) and was consequently proposed as a putative tumor suppressor gene (Tanaka et al., 1991). The Notch cascade plays a central role in developmental processes, controls many cell fate decisions and represents a pathway that regulates intestinal homeostasis (Stanger et al., 2005; van Es and Clevers, 2005). Multiple Notch cascade components are expressed in intestinal tissue, including the transcription factor gene *Hath1* (Artavanis-Tsakonas et al., 1999). Studies on *Hath1* have revealed a strong decrease in its expression in colon tumor tissues (Leow et al., 2005). Furthermore, expression analyses of colon carcinoma samples and normal mucous tissue have shown a higher expression of the γ-secretase substrates Ephrin-B2, GHR and TYRP2 (Liu et al., 2002; Yang et al., 2004; Ying-Tao et al., 2005), whereas expression of the substrate HLA-A2 was lost in a subset of colorectal carcinomas (Rees et al., 1988). These data thus imply that the γ-secretase pathway may represent a predisposition pathway for CRC susceptibility.

No significant association of the *APH1B* polymorphism was found with throat, prostate and lung cancer, the other three cancer subtypes studied. Nevertheless, all cohorts showed a higher prevalence of the risk allele in the patients, pointing to a disturbed regulation of a common signalling pathway involved in cancer pathogenesis. More detailed analyses in additional cancer case-control cohorts will learn whether the *APH1B* Phe217Leu polymorphism is indeed a risk factor for a wide variety of cancer subtypes.

Besides cancer and a number of neurodevelopmental disorders, also patients with rheumatoid arthritis and celiac disease had a higher prevalence of the Phe217Leu risk allele compared to their control populations. Rheumatoid arthritis and celiac disease are autoimmune diseases in which both genetic and environmental factors may play a role. As in other diseases with a complex aetiology, the genetic component of the two diseases is largely undefined. Our results may imply that *APH1B* constitutes a novel susceptibility gene for autoimmune disorders. Furthermore, celiac disease patients sometimes show neurological complications, such as epileptic seizures, and are thought to share predisposition factors for epilepsy (Gobbi, 2005; Grossman, 2008). Since we previously found association of the Phe217Leu allele with epilepsy (chapter B2) and we now find a higher prevalence of the Leu217 allele in celiac disease patients, we conclude that the Phe217Leu polymorphism may interfere with common signalling pathways for both epilepsy and celiac disease.

Since of the eleven disorders with a complex aetiology nine showed a higher prevalence of the *APH1B* Leu217 allele in the patients compared to the healthy control populations, together our data suggest that the risk allele may increase the susceptibility for developing multiple complex disorders. If indeed true, collecting a proper control group for studying this SNP will be challenging, because the chances are high that a considerable number of individuals carrying the SNP will not be free

of disease. Thus, the control risk allele frequencies we found may in fact constitute an overrepresentation.

In conclusion, the Phe217Leu polymorphism in the human *APH1B* gene may constitute a risk factor for the pathogenesis of a wide variety of disorders with a complex aetiology.

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General discussion

Disorders with a complex aetiology, including neurodevelopmental disorders, are thought to result from a combination of genetic and environmental factors. Although many genes and pathways have been suggested to be linked to disorders with a complex aetiology, the identities of the causal genes are mostly still unknown. The aim of the research described in this thesis was to provide a contribution to a better understanding of the genes and pathways involved in the pathogenesis of complex disorders, especially the neurodevelopmental disorders. To get a better insight into the genetic causes, we initially used the APO-SUS/-UNSUS rat model with features reminiscent of complex phenotypes and subsequently employed human case-control cohorts in genetic association analyses. In the following paragraphs, the major findings of these studies are discussed and placed into a broader context.

APO-SUS and –UNSUS rats and the Aph-1b locus

The APO-SUS/-UNSUS rats constitute an animal model with a complex phenotype, including features that are similar to those seen in patients with schizophrenia (Ellenbroek and Cools, 2002). Microarray analysis (mRNA expression profiling) of brain regions from APO-SUS and –UNSUS rats revealed only one reproducible difference between the two rat lines (**chapter A1**); APO-SUS rats exhibit lower *Aph-1b* mRNA levels when compared to those in APO-UNSUS rats. Interestingly, the difference in *Aph-1b* mRNA expression level was found in both the original APO-SUS/-UNSUS rat lines (breeding started in 1985) and the replicate APO-SUS/-UNSUS lines (started in 1995). The two breeding lines showed a similar distribution of the *Aph-1b* genotypes: three gene copies in APO-UNSUS rats and one or two gene copies in APO-SUS rats, indicating that the pharmacological selection that was used to establish the APO-SUS and –UNSUS lines had resulted in a similar genotypic distribution in both the original and replicate lines.

Along with single-nucleotide polymorphisms (SNPs), such differences in gene copy number (copy number variations; CNVs) are considered key genetic variations contributing to phenotypic variation, complex traits and disease. Mainly during the last decade, a number of CNVs have been identified that affect expression levels of genes critical for normal development and consequently are responsible for abnormal behavioural phenotypes. For example, a microdeletion of ~1.6 Mb on chromosome 7q11.23 results in Williams-Beuren syndrome (OMIM# 194050); a ~4 Mb deletion on chromosome 15q11-q13 in Angelman (OMIM# 105830) or Prader-Willi syndrome (OMIM# 176270); and deletion of 22q11.2 causes DiGeorge (OMIM# 188400) or velo-cardio-facial syndrome (OMIM# 192430).

Detailed genomic analysis of the rat *Aph-1b* locus (on chromosome 8q24) revealed a segmental duplication (also referred to as low-copy repeat) within the *Aph-1b* region, creating a genomic architectural structure favourable for rearrangements.

Rearrangements (duplication, deletions and inversions) especially occur if segmental duplications (i) are > 10 kb in size, (ii) have a >97% sequence identity, (iii) are oriented in the same direction and (iv) are within 5 Mb of each other (Lupski, 1998). Since the rat *Aph-1b* gene copies each span ~20 kb, have a high degree of identity surrounding exon 5 (> 98.2% identity over 2377 nucleotides), are oriented in the same direction and are separated by only ~24 kb, the *Aph-1b* locus in the rat is susceptible to rearrangement events.

Normal Wistar rats from the Nijmegen outbred population (the original rat source used for the APO-SUS/-UNSUS breeding) have three copies of the *Aph-1b* gene (**chapter A4**). Hence, the reduction in copy number in the APO-SUS rats in both original and replicate lines was likely induced during breeding of the rat lines. Such a genomic rearrangement could have been caused by the injection of the rats with the dopamine receptor agonist apomorphine. For the generation of the APO-SUS/-UNSUS lines, this compound was used to select Wistar rats with a low and high apomorphine susceptibility. Apomorphine and, more specifically, its oxidation derivative 8-oxo-apomorphine-semiquinone (8-OASQ), have been found to induce cytotoxic and genotoxic effects, including oxidative DNA damage and frameshift mutations (Moreira et al., 2003; Picada et al., 2003b; Picada et al., 2003a). By injecting the rats with this neurotoxic compound, the rearrangement event might have been triggered, leading to rats with reduced *Aph-1b* copies. To test whether the *Aph-1b* recombination event can indeed be induced by apomorphine, Wistar rats with a low and a high apomorphine susceptibility (all harbouring three copies of the *Aph-1b* gene) were injected with oxidized apomorphine for five consecutive days (1.5 mg/kg s.c.). A reduction in *Aph-1b* copy number was observed in the offspring of parents with high as well as parents with low apomorphine susceptibilities (data not shown). Thus, the *Aph-1b* recombination event can indeed be induced by oxidized apomorphine and appears to be independent of the degree of apomorphine susceptibility of the rats.

The apomorphine-induced recombination event in the *Aph-1b* locus suggests that other genomic regions could also be affected by (oxidized) apomorphine injections. Preliminary analysis of the genomes of the offspring of the apomorphine-injected rats showed two other genomic regions to be affected by the apomorphine injections (a CNV locus located within the *Ncam2* gene and a CNV locus located within the *Spna1* gene; for localisation of the CNVs, see **chapter A5**). It would be of interest to investigate whether a consensus sequence is shared by these genomic regions. Such a consensus may represent a hot spot for apomorphine-induced genomic rearrangements. Of note is the fact that subcutaneous injections of apomorphine are also performed in humans (for example for the treatment of Parkinson's disease and erectile dysfunction), even upto 200 mg/day (=2.5 mg/kg based on an individual

of ~80 kg). It is thus worthwhile to further investigate the effect of (oxidized) apomorphine on human genome stability.

Is Aph-1b the only causative factor of the complex APO-SUS phenotype?

If the gene-dosage imbalance of the *Aph-1b* gene is the only causative factor for the phenotypical differences between the APO-SUS and –UNSUS rats, the number of *Aph-1b* gene copies (1, 2 or 3) should segregate with the APO-SUS/-UNSUS characteristics. **Chapter A1** indeed shows a gene-dosage effect for some APO-SUS/-UNSUS-related features (e.g. locomotor activity in the open field and open arm entries in the elevated plus maze). Yet, for a detailed gene-dosage dependency of the complex phenotypical features, crossbreeding experiments are necessary, such that the genetic background of the APO-SUS and –UNSUS rats is first mixed and subsequently reselected for the *Aph-1b* genotypes. If, following crossbreeding, the number of *Aph-1b* gene copies is still linked to the APO-SUS/-UNSUS-specific features, *Aph-1b* should be considered the causal factor of the complex APO-SUS phenotype.

As described in **chapter A2**, APO-SUS rats with one copy of the *Aph-1b* gene (I/I) were crossed with APO-UNSUS rats with three copies (III/III). Using a reselection procedure based on the *Aph-1b* genotypes, animals were obtained with a genetically identical background, except for the *Aph-1b* locus (termed *Aph-1b* I/I and *Aph-1b* III/III rats). Analysis of the apomorphine susceptibility of the *Aph-1b* I/I and III/III rats showed in both rat lines individuals with low and high gnawing scores (figure 1). If the number of *Aph-1b* gene copies would be responsible for the apomorphine susceptibility, *Aph-1b* I/I rats should have shown gnawing scores as observed in APO-SUS rats (>500 counts/45 min) and *Aph-1b* III/III rats as seen in APO-UNSUS rats (<10 counts/45 min). Since we now find individuals with low and high gnawing scores in both the *Aph-1b* I/I and III/III lines, we conclude that the number of *Aph-1b* gene copies is not directly linked to apomorphine susceptibility. In addition, analysis of the *Aph-1b* I/I and III/III rats for their exploratory behaviour on the open field and elevated plus maze also failed to show a correlation with the number of *Aph-1b* gene copies (data not shown). Since apomorphine susceptibility and exploratory behaviour represent two phenotypes that are different between APO-SUS and -UNSUS rats, *Aph-1b* is likely not the only genetic factor determining the difference between the two rat lines. Nevertheless, these data do not exclude the possibility that an interplay of *Aph-1b* with some other (epi)genetic factor is responsible for the characteristics seen in the APO-SUS/-UNSUS model. Such a factor would then have been mixed among the various rats during the crossbreeding of the animals.

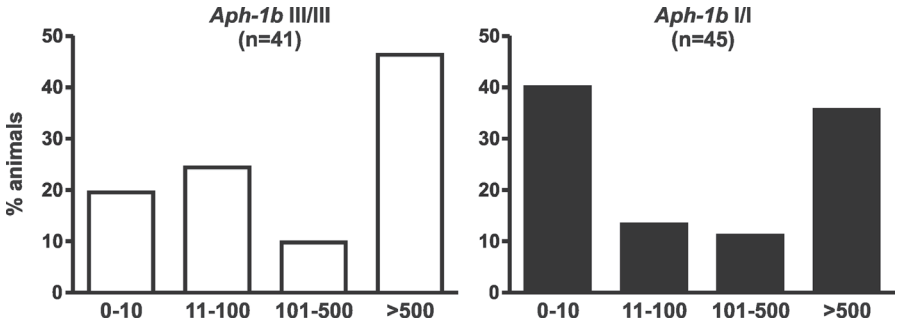

Figure 1. *Distribution of the gnawing scores in Aph-1b III/III and Aph-1b I/I rats following 1.5 mg/kg apomorphine injection (s.c.). The percentages of animals are scored based on the gnawing counts per 45 minutes: 0-10, 11-100, 101-500 and more than 500 times.*

Other genetic variations in APO-SUS and –UNSUS rats

Our recent experiments thus suggest that the *Aph-1b* gene-dosage imbalance is not solely responsible for the complex APO-SUS phenotype. As shown in **chapters A4** and **A5** more genetic and also epigenetic variations are present between the APO-SUS and –UNSUS rats, with similar (epi)genetic distributions in both the original and replicate lines. We observed at least five additional genetic variations (**chapter A4**), one epigenetic variation (**chapter A4**) and eight CNVs (CNVs located on chromosomes 1, 5, 10, 11 and 17; **chapter A5**) between the APO-SUS and –UNSUS (epi)genomes. Since rat CNVs have been found to overlap with CNV regions in human (Guryev et al., 2008), our findings may well be of significance for understanding complex human disorders.

Preliminary SNP analysis of the genomes of the replicate APO-SUS and –UNSUS rat lines (sequencing analysis of ~288.000 bp per rat genome) showed that in the coding regions of the APO-SUS and –UNSUS genomes one SNP exists in every ~7 kb (in collaboration with Prof. Dr. E. Cuppen, Hubrecht Institute, Utrecht, The Netherlands; data not shown). When analysing also the non-coding regions, an even higher SNP frequency would probably be observed. Given the fact that approximately 2.75 billion base pairs constitute the rat genome (Gibbs et al., 2004), the genomes of APO-SUS and –UNSUS rats are expected to contain at least 400.000 SNPs. In general, it appears that two individuals from the Wistar population are genetically closer than APO-SUS and –UNSUS rats.

A number of the (epi)genetic variations (SNPs, CNVs and DNA methylation variations) may thus contribute to the phenotypical differences observed between APO-SUS and –UNSUS rats. For a better understanding of the significance of any

of the genetic differences in the complex APO-SUS phenotype, it may be helpful to analyse the (epi)genetic variations in phenotypically well-defined Wistar rats (e.g. Wistar rats with known apomorphine susceptibility levels, locomotor activity in the open field and open arm entries in the elevated plus maze). If a (epi)genetic variation is found to be associated with any of the traits, then this (epi)genetic variation should be considered a factor presumably involved in the complex APO-SUS phenotype. For a more definitive link between any of the (epi)genetic differences and the complex APO-SUS phenotype, it will also be necessary to perform crossbreeding experiments, with a subsequent reselection for the (epi)genetic variation, similar as we have been performing for the *Aph-1b* gene-dosage imbalance. Detailed phenotypical analysis of the new lines will be necessary to understand the functional consequence of the (epi)genetic variation. The complex APO-SUS phenotype may also be the result of multiple genetic alterations instead of only a single-gene variation. Therefore it might be interesting to examine whether combinations of genetic variations (SNPs, CNVs and DNA methylation variations) are linked to the complex APO-SUS phenotype.

Despite the fact that many genetic variations were found between the APO-SUS and –UNSUS rats (**chapters A4** and **A5**), only one reproducible difference at the mRNA expression level was observed when comparing the gene expression profiles of the two rat lines (**chapter A1**). A number of possibilities may explain this surprising situation. First, at that time, only one third of all gene transcripts were represented on the microarrays. Moreover, the detection sensitivity of the arrays was rather limited. To allow detection above background, mRNA expression differences of at least ~1.4-fold were necessary. Hence, additional APO-SUS/-UNSUS-specific mRNA expression differences may have been missed because they represented only small expression differences or the corresponding gene transcripts were not represented on the arrays. Application of one of the currently available microarray platforms with most genes represented and with a relatively high resolution may lead to the identification of new genes and pathways that underlie the differences in the APO-SUS/-UNSUS phenotypes.

Second, in our microarray analysis hippocampal tissue was used. The hippocampus is a relatively large structure with many different cell types. By analysing the whole hippocampus one may thus not detect an expression difference that is present in only a subset of cells. Isolation of a specific hippocampal region consisting of a smaller subset of cells (e.g. CA1, CA3 or dentate gyrus) may increase the chances of finding APO-SUS/-UNSUS-specific differences. Furthermore, analysis of brain regions other than the hippocampus (e.g prefrontal cortex, nucleus accumbens (ventral striatum), caudate-putamen (dorsal striatum), amygdala, ventral tegmental area, substantia nigra pars compacta and hypothalamus) may give new insights into the genes and pathways linked to the complex APO-SUS phenotype.

Third, brain tissues from postnatal day 9 and 60 rats were used. Future studies may include mRNA expression profiling in other, including earlier, developmental stages. Histopathological findings in schizophrenia patients, such as dilation of the lateral ventricles (Johnstone et al., 1976), a decreased brain weight, length and volume (Brown et al., 1986; Pakkenberg, 1987; Bruton et al., 1990), a decreased volume of the thalamus (Pakkenberg, 1990) and enlarged basal ganglia (Heckers et al., 1991), are thought to arise early in development. Thus, the causal processes for schizophrenia pathogenesis are present long before the diagnosis is actually made. Such a neurodevelopmental hypothesis for the background of complex disorders would therefore ask for analysis of embryonic stages rather than of time points later in life. Future spatial and temporal expression analysis may reveal whether additional mRNA expression differences exist and whether they have a functional contribution to the APO-SUS/-UNSUS-specific features.

The human APH1B gene and complex human disorders

In contrast to rodents, humans have only one copy of the *APH1B* gene (located on chromosome 15q22.2), so an unequal crossing-over event of the *APH1B* gene cannot occur. Preliminary Southern blot analysis of schizophrenic patients and healthy controls did not reveal any duplications, deletions or rearrangements within the *APH1B* locus (data not shown). Interestingly, BLAST analyses revealed the presence of exons 1, 5 and 6 of the *APH1B* gene (without any intronic sequences) on chromosome 7q21.11. This chromosomal region may be considered a so-called processed pseudogene likely derived from alternatively spliced *APH1B* mRNA that was subsequently reverse transcribed and inserted into the nuclear genome. Although the biological effect of pseudogenes is still unclear, it has been shown that at least 20% of the pseudogenes is transcribed (either by using its own promoter or by using the promoter of a nearby gene) (Zheng et al., 2007). In addition, pseudogenes have been found to regulate the expression of its homologues (Hirotsune et al., 2003). To explore if the *APH1B* pseudogene regulates the expression of the intact *APH1B* on chromosome 15, future studies on this pseudogene in both patients and controls are warranted (e.g. expression analysis and mutation analysis).

Besides a gene-dosage imbalance as seen in the rat, other variations could influence *APH1B* mRNA and protein expression levels as well, such as small insertions and deletions, SNPs, mutations and also epigenetic alterations. In the human *APH1B* gene (including the 5'- and 3'-untranslated regions (UTRs)), 151 SNPs have been identified (based on NCBI SNP database). Non-synonymous SNPs that lead to an amino acid change are of special interest, since a considerable amount of non-synonymous SNPs have been linked to human diseases (Krawczak et al., 2000). To date, in the commonly used SNP databases (NCBI, Ensembl and HapMap databases)

only one non-synonymous *APH1B* SNP (Phe217Leu; rs1047552) has been recorded as being validated (by multiple independent submissions to the refSNP cluster).
The Phe217Leu polymorphism was found to be a risk factor for premature coronary atherosclerosis (**chapter B1**), epilepsy (**chapter B2**), HIV-1 infection (**chapter B3**), colorectal cancer (**chapter B4**) and Alzheimer's disease (AD) (Poli et al., 2003; Poli et al., 2008). In addition, of the eleven additional complex disorders examined nine showed a higher prevalence of the risk allele in the patients compared to the healthy control populations (**chapter B4** and table 1), which is higher than expected by chance.
Interestingly, some of the human disorders showing a significant association with the Phe217Leu SNP had phenotypical traits similar to those found in the APO-SUS/-UNSUS model (table 2): (i) APO-SUS rats display an increased incidence of bursts of bilateral synchronous spike wave discharges (de Bruin et al., 2000), as can be seen in patients with epilepsy (Hughes and Fino, 2004), (ii) APO-SUS rats display an impaired vasorelaxation to adrenergic stimuli, increasing the risk for the development of hypertension and vascular diseases such as atherosclerosis (Smits et al., 2002; Riksen et al., 2003), and (iii) APO-SUS rats display a stronger T helper 2 (Th2)-dependent IgE response upon worm infection, a lower Th1-mediated autoimmune encephalomyelitis response and, like in patients with HIV-1 infection, a disturbed Th1/Th2 balance (Kavelaars et al., 1997). Thus, a subtle effect on *APH1B* expression or function (*Aph-1b* copy number variation in rat or *APH1B* Phe217Leu variation in human, respectively) may be involved in a number of complex traits and diseases (figure 2).

γ-Secretase signalling in complex disorders

Together with its paralogue Aph-1a, Aph-1b is part of the γ-secretase enzyme complex. Aph-1 (either Aph-1a or -1b) can form with presenilin (either PS1 or PS2), nicastrin and presenilin enhancer 2 (Pen2) a functional γ-secretase enzyme complex that can cleave many type I transmembrane proteins within their membrane domain. Upon cleavage, the active cytoplasmic domains can (i) travel to the nucleus where they may regulate gene expression (Okamoto et al., 2001; LaVoie and Selkoe, 2003; Marambaud et al., 2003) or (ii) bind to their partners in the cytoplasm to exert their specific functions (Schulz et al., 2003). As reviewed recently, at least 30 γ-secretase substrates have been identified; amyloid-β (Aβ) precursor protein (APP), Aβ precursor-like protein 1 and 2 (APLP1 and APLP2), low density lipoprotein-related protein 1, 2 and 8 (LRP1, LRP2 (Megalin) and LRP8 (APOER2)), N- and E-cadherin, γ-Protocadherin, Notch1-4 and the ligands Delta1 and Jagged, Neuregulin-1 and -2 (NRG1 and 2), ErbB4, Sialophorin (CD43), CD44, colony stimulating factor 1 (CSF1), deleted in colorectal carcinoma (DCC), Ephrin-B1 and -B2, growth

Table 1. *APH1B* Phe217Leu variation in a number of diseases with a complex aetiology

Disorder	Country	MAF controls (%)	MAF patients (%)	OR	*p*	RR ↑ or ↓
Neurodevelopmental disorders						
Schizophrenia	Norway	2.6	3.4	1.32	0.55	↑
Schizophrenia	Sweden	3.6	4.1	1.16	0.66	↑
Schizophrenia	Sweden	2.6	2.7	1.05	0.88	↑
Schizophrenia	Denmark	2.6	2.1	0.80	0.45	↓
Schizophrenia	The Netherlands	1.4	1.6	1.15	0.76	↑
Schizophrenia	Belgium	2.4	1.7	0.71	0.36	↓
Schizophrenia	USA	3.7	3.7	1.00	0.99	=
Epilepsy	The Netherlands	1.4	3.4	2.42	0.048	↑
Epilepsy	UK	2.4	2.9	1.19	0.65	↑
Bipolar disorder	Norway	2.6	3.4	1.34	0.55	↑
Bipolar disorder	Belgium	2.4	6.7	2.86	0.041	↑
Autism	Belgium	2.4	5.1	2.15	0.073	↑
ADHD	The Netherlands	1.4	2.4	1.68	0.50	↑
Dyslexia	The Netherlands	1.4	2.2	1.55	0.51	↑
Depression	USA	3.7	3.2	0.86	0.63	↓
Cancer						
Colorectal cancer	The Netherlands	1.4	3.6	2.55	0.0021	↑
Colorectal cancer	The Netherlands	2.1	2.6	1.20	0.36	↑
Throat cancer	The Netherlands	1.4	2.6	1.86	0.079	↑
Prostate cancer	Australia	2.6	3.1	1.21	0.39	↑
Lung cancer	Australia	2.6	2.9	1.13	0.56	↑
Other complex diseases						
Premature atherosclerosis	The Netherlands	2.0	2.7	1.35	0.15	↑
HIV-1 infection	The Netherlands	2.2	3.6	1.67	0.049	↑
HIV-1 infection	South Africa	2.2	5.1	2.33	0.087	↑
Rheumatoid arthritis	Sweden	1.8	2.3	1.30	0.45	↑
Celiac disease	The Netherlands	1.4	2.0	1.38	0.46	↑
Alzheimer's disease*	Italy	1.4	3.2	2.34	0.01	↑

*MAF = minor allele frequency; OR = odds ratio; RR = relative risk; * = Poli et al., 2008.*

hormone receptor (GHR), glutamate receptor 3 (GluR3), human leucocyte antigen-A2 (HLA-A2), Nectin-1α, neurotrophin receptor associated death domain (NRADD), nerve growth factor receptor ($p75^{NTR}$), sodium channel, voltage-gated, type II, beta

(Scnb2), Alcadein α-γ, Syndecan-3, Tyrosinase (TYR) and tyrosinase-related protein 1 and 2 (TYRP1 and TYRP2) (Kopan and Ilagan, 2004; Parks and Curtis, 2007). Because for most γ-secretase substrates the biological relevance of intramembrane cleavage is still unclear, most cellular roles that are fulfilled by γ-secretase are not yet understood. Nevertheless, it is clear that with every new substrate discovered, the physiological relevance of the γ-secretase complex widens.

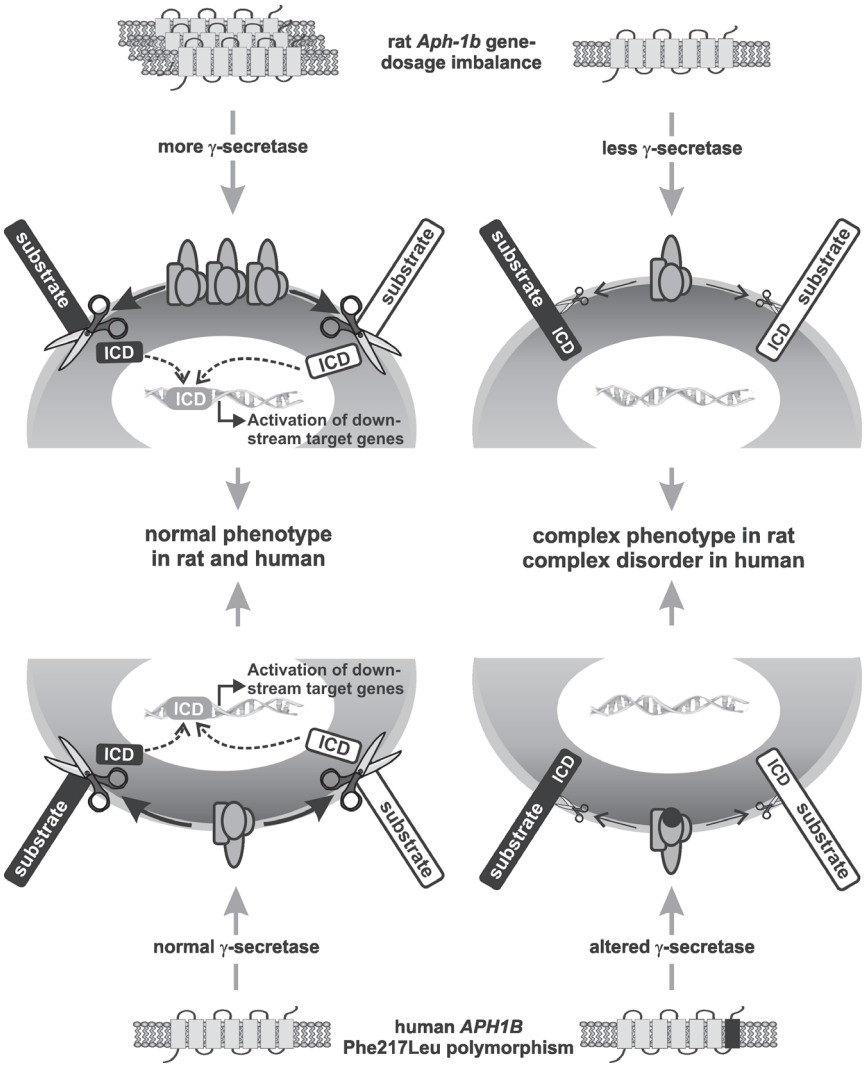

Figure 2. *Model of affected γ-secretase signalling in rat and human. The affected expression or function of Aph-1b (Aph-1b copy number variation in rat and Phe217Leu variation in human, respectively) can lead to substrate-specific alterations in γ-secretase cleavage activity. Subsequent differences in the amount of released intracellular domain (ICD) may result in complex traits or diseases.*

For understanding AD, the γ-secretase complex has long been of interest since it cleaves the AD-related APP protein. Since we now find a possible link of the γ-secretase complex with a number of complex traits, γ-secretase signalling may not only be involved in AD, but also in a variety of other diseases (atherosclerosis, epilepsy, HIV-1 infection and colorectal cancer; **chapters B1-B4**). Interestingly, a number of γ-secretase substrates have been reported to be implicated in the pathogenesis of one or more of these associated diseases (table 2). Obviously, further studies are required to completely understand the role of γ-secretase and its substrates in the pathogenesis of disorders with a complex aetiology.

Complicating factors in association studies for disorders with a complex aetiology

Although a number of significant associations have been found, it is clear that our human genetic associations ask for replication in additional cohorts to understand the contribution of the γ-secretase signalling pathway to the pathogenesis of complex disorders. Unfortunately, such genetic association analyses for complex disorders are greatly complicated by a number of factors that are described below.

Population stratification

Genetic variations often occur among (geographically or ethnically) different populations and this fact may thus increase the difficulty in data interpretation as well. For correct stratification and successful replication, it is therefore highly important that samples are clinically, geographically and ethnically well characterized.

Definition of phenotype

In the genetics of psychiatric disorders, the definition of a phenotype is one of the main problems. Most genetic studies use patient characterization according to the Diagnostic and Statistical manual for mental disorders version IV (DSM-IV, 2000) or the International Classification of Disease version 10 (ICD-10, 1992) criteria. However, investigators nowadays believe that the phenotype should be specified in more detail, since most neurodevelopmental disorders include a number of intermediate clinical subtypes and distinct phenotypical parameters (endophenotypes) (Gottesman and Gould, 2003), presumably each with a different genetic background. Such endophenotypes may help in the identification of risk factors, although the effectiveness of this approach has recently been questioned (Flint and Munafo, 2007). Nevertheless, analysis of an endophenotypically defined group of patients may increase replication efficacy.

Table 2. Risk effects for human *APH1B* Phe217Leu variation and rat *Aph-1b* gene-dosage imbalance

human *APH1B* Phe217Leu variation	rat *Aph-1b* gene-dosage imbalance	γ-secretase substrates possibly involved in complex trait or disease
↑↓ risk for developing a neurodevelopmental disorder (NS; **chapter B4**)	↑ risk for neurodevelopmental features (reviewed by Ellenbroek and Cools, 2002)	APP (Sokol et al., 2006) DCC (Flores et al., 2005) ErbB4 (reviewed by Mei and Xiong, 2008) GluR3 (Ibrahim et al., 2000) HLA-A2 (Ozcan et al., 1996) LRP8 (Barr et al., 2007) Notch4 (Wei and Hemmings, 2000) NRG 1 (reviewed by Mei and Xiong, 2008) $P75^{NTR}$ (Rajakumar et al., 2004)
↑ risk for developing epilepsy (**chapter B2**)	↑ incidence of bursts of bilateral synchronous spike wave discharges (de Bruin et al., 2000)	GluR3 (Rogers et al., 1994) HLA-A2 (Fontana et al., 1978) Scnb2 (Chen et al., 2002)
↑ risk for developing premature coronary atherosclerosis (male-specific association; **chapter B1**)	↑ risk for developing atherosclerosis due to reduced relaxation of mesenteric arteries upon α_2 and β_2-agonist stimulation (Smits et al., 2002; Riksen et al., 2003)	APP (Nelson and Alkon, 2005) CD44 (Vendrov et al., 2006) CSF-1 (Rajavashisth et al., 1998) Ephrin-B1 and -B2 (Sakamoto et al., 2008) ErbB4 (Gassmann et al., 1995) LRP (Hiltunen et al., 1998; Schulz et al., 2002) NRG (Clement et al., 2007)
↑ risk for HIV-1 infection (**chapter B3**)	↑ T_{H2} response upon infection and a disturbed Th1/Th2 balance (Kavelaars et al., 1997)	CD43 (Barat and Tremblay, 2002) HLA-A2 (Papasteriades et al., 1993) Syndecan-3 (Bobardt et al., 2003)
↑ risk for developing colorectal cancer (**chapter B4**)	↓ angiogenesis and tumor growth (Teunis et al., 2002)	DCC (Fearon et al., 1990; Itoh et al., 1993) Ephrin-B1 and B2 (Tang et al., 1999; Liu et al., 2002) GHR (Yang et al., 2004) HLA-A2 (Rees et al., 1988) Notch (Stanger et al., 2005; van Es and Clevers, 2005) TYRP2 (Ying-Tao et al., 2005)
↑ risk for developing rheumatoid arthritis (NS; **chapter B4**)	↓ sensitivity for rheumatoid arthritis (van de Langerijt et al., 1994)	CD43 and CD44 (Humbria et al., 1994) CSF1 (Cupp et al., 2007) Ephrin-B1 (Kitamura et al., 2008) HLA-A2 (Ford et al., 1984) LRP2 (Ooka et al., 2003) Notch and the ligands Delta1 and Jagged (Nakazawa et al., 2001; Yabe et al., 2005) Syndecan-3 (Patterson et al., 2005)
↑ risk for celiac disease (NS; **chapter B4**)	-	CD44 (Kemppainen et al., 2005) CSF1 (Desreumaux et al., 1998) E-cadherin (Perry et al., 1999) HLA-A2 (Kedzierska and Turowski, 2000)

NS: not significant

Gene-environment interactions
Another complicating factor in the identification of susceptibility genes for complex disorders concerns the possibility of multifactorial gene-plus-environment interactions. Unfortunately, such interactions are still difficult to quantify and interpret.

Multiple genes hypothesis
The search for susceptibility genes is further complicated when the aetiology of a complex neurodevelopmental disorder can not be explained by a single genetic variant with a relatively large effect but is rather caused by an interplay of a number of genes with small (additive) effects. For the complex disorders, the essential parameters for single- and multiple-locus models have been calculated and an interaction of about three different genes together with the environment was predicted to underly this disorder (Risch, 1990). Not surprisingly, in general the greater the number of genes involved the more difficult their identification will be and larger (or additional) cohorts will be necessary to reach significant association. In addition, besides the possibility that multiple susceptibility genes are involved, individuals may be affected by the absence of protective alleles, while epistasis may also play a role.

Common disease-common variant or rare-variant hypothesis
At present, it is not clear whether only a relatively small number of common genetic variants are linked to the aetiology of complex disorders (known as the "common disease-common variant hypothesis", often abbreviated CD-CV) (Reich and Lander, 2001; Peng and Kimmel, 2007), or if a large number of rare genetic variants is involved ("rare-variant-hypothesis" or heterogeneity hypothesis) (Pritchard, 2001). In case of CD-CV, association analyses (e.g. GWA studies) may detect genetic variants if the studies contain enough power. It is clear that association analyses will be more difficult in rare-variant cases (Slager et al., 2000).

Future perspectives
The data described in this thesis illustrate the way science often works. While new insights concerning the genetics of complex disorders have been obtained, our results lead to new opportunities and hypotheses for further research. Our data implicate that diseases with a complex aetiology may be the result of an affected γ-secretase signaling. However, additional (epi)genetic factors will be involved as well.
The APO-SUS/-UNSUS rat model provides an interesting model to analyze the genetic background of disorders with a complex aetiology. The precise molecular basis underlying the model remains to be elucidated. Extensive genome-wide genetic screening (for example by using high throughput SNP array analysis or complete

sequencing of the two genomes) will be valuable to get a better insight into the genetic differences between the two rat lines. Furthermore, genome-wide tissue- and time-specific mRNA and protein expression analyses should be performed (for example by using microarray analyses or 2D-gel electrophoresis on blood, spinal fluid or brain tissues). Besides differences at the mRNA and protein expression levels, changes in microRNA (miRNA) levels have also been found to correlate with diseases (reviewed by Guarnieri and DiLeone, 2008). Currently, microarray chips for rat miRNA expression profiling are on the market and might thus be instrumental to examine whether miRNAs are involved in the development of the complex APO-SUS phenotype.

Besides genetic and expression analyses, it might be worthwhile to obtain more detailed insights into the anatomical differences between the brains of APO-SUS and –UNSUS rats. For this purpose, histological stainings of the brain may be helpful. Furthermore, neuroimaging (e.g. functional magnetic resonance imaging (fMRI) or positron emission tomography (PET)) may help assessing any differences in APO-SUS and –UNSUS brain functioning. Together, such approaches will be helpful to identify new genes and pathways involved in a specific complex trait. Alternatively, tracking down behavioral outliers within the APO-SUS and –UNSUS populations (e.g. APO-SUS rats with low apomorphine susceptibilities or APO-UNSUS rats with high apomorphine susceptibilities), and subsequent analysis of the parameters described above may be rewarding.

Regarding the gene * environment interactions that clearly occur in the APO-SUS/-UNSUS model system (Ellenbroek et al., 2000; Degen et al., 2004), it might be interesting to study the effect of early-life experiences (e.g. maternal deprivation or cross-fostering) on the behavioral abnormalities in adulthood. Subsequent genetic and expression profiling may be a fruitful approach to further investigate the genes and molecular pathways involved. We expect that the degree of maternal care may influence the degree of vulnerability to develop behavioral abnormalities later in life. Upon exposure to a stressful experience during adulthood, the abnormalities may become apparent (so-called three hit model of stress-induced psychopathology; a disease results from an interaction between (i) genetic factors, (ii) early-life events and (iii) late environmental factors (Ellenbroek, 2003; Ellenbroek et al., 2005)). Therefore, it will be interesting to see whether APO-SUS and –UNSUS rats exposed to early-life events and late environmental stressors show differences in their behavioral phenotypes and whether this is coupled to their genetic/molecular build-up.

The objective of using the APO-SUS/-UNSUS model is to translate the results by validating the susceptibility pathways in human. The data from our human association studies have to be replicated and should be followed up to understand the

contribution of γ-secretase to disorders with a complex aetiology. In addition, future studies may include *Aph-1b* Phe217Leu analysis in complex disorders that have not been tested in our studies (e.g. mental retardation, addiction, anxiety disorders and posttraumatic stress disorder). For a better understanding of the aetiologies involved in complex disorder pathogenesis, it will be helpful to obtain detailed clinical, ethnical and geographical information on large groups of individuals. In addition, the environmental factors need to be well defined and documented. In general, knowledge of the relevant environmental risk factors is rather limited. Close collaborations between psychiatrists and genetic researchers are therefore required.
Currently, the first genome-wide association studies with high-throughput SNP arrays have been reported for a number of complex disorders (Steer et al., 2007; Baum et al., 2008; Meaburn et al., 2008). Undoubtedly, in the near future many more studies with the 500K and even larger SNP arrays can be expected. Such studies will however not cover all genetic variations in the genome (Wollstein et al., 2007), because SNPs with a low minor allele frequency (MAF) (<0.05) are usually not included on the arrays, thereby excluding analysis of rare genetic variants, like the *Aph-1b* Phe217Leu SNP. This is unfortunate, since the rare genetic variants are generally considered to have a higher chance of being causative (Cargill et al., 1999). Thus, candidate gene approaches on selected SNPs with a low prevalence may increase the chances to identify functional genetic variants. Because one would expect that causal SNPs have an effect in any population, a further consideration may involve a choice of SNPs with a low MAF in all ethnical populations. In this connection, one has to be aware of the possibility that such a SNP may need an additional polymorphism(s) to explain the phenotype (multiple genes hypothesis), while the additional genetic variation(s) may not be present in a particular ethnical population. One of the practical problems in dealing with low-MAF SNPs concerns the sample size necessary to obtain reliable association data, i.e. the lower the MAF the more samples are required to reach statistical significance. It is likely that inclusion of potentially functional SNPs (non-synonymous SNPs and SNPs in gene promoter regions or exon-intron boundaries) will increase the success rate in the analysis.

Clearly, many questions remain unanswered with respect to the pathogenesis of disorders with a complex aetiology. Nevertheless, it is to be expected that within the next years the tsunami of genetic research, in combination with the use of well-defined animal models, will result in the identification of susceptibility genes for these disorders. Eventually, the acquired understanding of the molecular mechanisms underlying complex disorders may lead to translational research, including the design of gene/pathway-specific drugs and the application of disease-preventing strategies.

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Summary

Summary

Nearly all diseases have a genetic component. Some diseases are caused by alterations in a single gene (monogenic diseases), but for most conditions and diseases the causes are much more complex since they are thought to result from a combination of (more than one) genetic and environmental factors. The group of these so-called complex disorders (or multifactorial disorders) include diseases like asthma, diabetes, heart disease, rheumatoid arthritis, hypertension, atherosclerosis and cancer, the neurodegenerative diseases (such as Alzheimer's disease, AD) and the neurodevelopmental disorders (e.g. schizophrenia, attention deficit hyperactivity disorder-ADHD and autism). Complex disorders often cluster in families, but have a relatively low heritability when compared with the monogenic diseases. Due to the low level of inheritance, the influence of environmental factors and the possibility of an interplay of a number of genes (instead of a single gene), it is often still elusive which gene (or genes) is responsible for the pathogenesis of these disorders. Therefore, treatment of complex disorders is usually difficult and mostly based on reducing the symptoms instead of curing the disease. A better understanding of the genes and pathways involved in complex disorders may be useful for the development of specific medicines and the application of disease-preventing strategies.

The objective of this thesis was to provide a contribution to a better understanding of the genes and pathways involved in complex disorders. An animal model may be helpful to further understand such genes and pathways. An example of an animal model for disorders with a complex aetiology constitutes the apomorphine-susceptible (APO-SUS) and apomorphine-unsusceptible (APO-UNSUS) rats. Since both genetic and environmental factors determine the phenotype of the APO-SUS/-UNSUS rats and APO-SUS rats display a number of behavioural and pathophysiological features reminiscent of complex disorders, the model may contribute to an analysis of the genetic background of complex disorders. Initially, we analysed the APO-SUS/-UNSUS rat model (part A) and subsequently translated our results by validating susceptibility pathways in human (part B).

In chapter A1, microarray analysis (mRNA profiling) of the hippocampus from APO-SUS and –UNSUS rats revealed a reduced expression of *Aph-1b* in APO-SUS rats when compared to APO-UNSUS rats. We observed that the difference in *Aph-1b* mRNA expression was found in the original as well as in an independent replication of the APO-SUS and –UNSUS rat lines (the lines were developed with a ten-year interval). Subsequent genomic analysis of the *Aph-1b* locus in the APO-SUS and –UNSUS rats revealed a gene-dosage imbalance; APO-SUS rats have one or two *Aph-1b* gene copies, whereas APO-UNSUS rats harbour three genes. The reduced *Aph-1b* mRNA expression in APO-SUS rats segregated with the reduced number of

Aph-1b gene copies. Aph-1b is a component of the γ-secretase enzyme complex that is responsible for the proteolytic processing of a wide variety of type I transmembrane proteins (including the amyloid-β (Aβ) precursor protein APP, neuregulin and Notch1-4) and is involved in multiple (neuro)developmental signalling pathways. The expression levels of the other γ-secretase components (Aph-1a, presenilin 1 and 2, nicastrin and presenilin enhancer 2) were not altered, whereas γ-secretase cleavage activity towards a number of substrates was found to be different between the APO-SUS and –UNSUS rats. Furthermore, the number of *Aph-1b* gene copies segregated with a number of behavioural traits, including apomorphine susceptibility, locomotor activity in the open field and open arm entries in the elevated plus maze.

Chapters A2 and A3 further describe the affected *Aph-1b* expression and γ-secretase cleavage activity in the APO-SUS/-UNSUS rat model. Expression levels of *Aph-1b* and its paralogues *Aph-1aS* and *-1aL* were investigated during development (from embryonic day 13 to postnatal day 100). These analyses revealed gene-dosage dependent differences in *Aph-1b* mRNA expression throughout development, whereas expression levels for *Aph-1a* were neither prenatally nor postnatally affected. Furthermore, we observed tissue-specific alterations in γ-secretase cleavage activity towards the APP family (APP and its relatives the APP-like proteins APLP1 and APLP2), $p75^{NTR}$, ErbB4 and neuregulin-2. We may thus conclude that when compared to the APO-UNSUS rats and throughout the entire developmental period the relatively low levels of *Aph-1b* mRNA and low γ-secretase activity in the APO-SUS rats likely affected (neuro)developmental signalling pathways and, consequently, contributed to the development of the complex APO-SUS phenotype. Nevertheless, experiments based on crossing, genetic re-selection and subsequent phenotypical analysis revealed that the *Aph-1b* gene-dosage imbalance is not the only causative factor for the phenotypical differences between the APO-SUS and –UNSUS rats.

In chapters A4 and A5, we identified in both the original and replicate APO-SUS/-UNSUS rat lines a number of genetic variations other than in *Aph-1b*. We observed eight copy number variations, five small genetic variations and one epigenetic variation (DNA methylation) in the APO-SUS and –UNSUS rat genomes. Together with the *Aph-1b* gene defect, a number of these newly identified variations may thus contribute to the complex APO-SUS phenotype.

The objective of using the APO-SUS/-UNSUS model was to translate the results by validating susceptibility pathways in human. We therefore performed human association studies to examine whether γ-secretase signalling is involved in the pathogenesis of disorders with a complex aetiology. In chapter B1 we showed that a non-synonymous single-nucleotide polymorphism (SNP) in the human *APH1B* gene (presence of a leucine instead of the evolutionarily conserved residue Phe217) causes a reduced γ-secretase cleavage activity towards one of its substrates, syndecan-3. Our

association analyses in a number of complex disorders showed a link between this SNP and disorders with a complex aetiology. We may thus assume that the human Phe217Leu polymorphism in the *APH1B* gene has a functional effect. Furthermore, we show a male-specific association of this *APH1B* SNP with premature atherosclerosis (chapter B1), as well as its association with epileptic seizures (chapter B2), HIV-1 infection (chapter B3) and colorectal cancer (CRC; chapter B4). In addition, a higher prevalence of the risk allele was observed in patients with bipolar disorder, autism, ADHD, dyslexia, rheumatoid arthritis, celiac disease, throat cancer, prostate cancer and lung cancer. However, these latter associations proved not to be statistically different from the respective controls, probably due to the low number of cases and the low frequency of the risk allele (chapter B4). Thus, the γ-secretase pathway may play a role in the pathogenesis of a number of complex disorders, suggesting that a subset of disorders with a complex aetiology have a common biological background.

Overall, the data presented in this thesis show that alterations in γ-secretase cleavage may contribute to the pathogenesis of a number of complex traits and disorders.

Samenvatting

Samenvatting

Bij het ontstaan van vrijwel alle ziekten is een genetische component betrokken. Sommige ziekten ontstaan door een defect in één enkel gen (monogenetische ziekten), maar voor de meeste ziekten en aandoeningen is de oorzaak veel complexer. De meeste ziekten worden namelijk veroorzaakt door een complex samenspel van een (of meerdere) gen(en) met omgevingsfactoren. Tot de groep van deze zogenaamde complexe ziekten (ook wel multifactoriële ziekten genoemd) behoren ziekten zoals astma, diabetes, hartfalen, hypertensie (hoge bloeddruk), reumatoïde artritis, atherosclerose, kanker, de neurodegeneratieve ziekten (zoals de ziekte van Alzheimer) en de (hersen)ontwikkelingsstoornissen (zoals schizofrenie, aandachtstekort/ hyperactiviteitstoornis-ADHD en autisme). Hoewel complexe ziekten vaak voorkomen in familieverband vertonen ze, in vergelijking met de monogenetische ziekten, een geringere mate van overerving. Vanwege deze lagere overervingsfactor, alsmede de invloed van omgevingsfactoren en de mogelijkheid dat een aantal genen samen (in plaats van één enkel gen) leiden tot de ziekte, is het vaak nog niet bekend welk gen (of welke genen) betrokken is bij de totstandkoming van een complexe ziekte. Zodoende is de behandeling van patiënten met een complexe ziekte vaak erg moeilijk en meestal slechts gebaseerd op het verminderen van de symptomen in plaats van het genezen van de ziekte. Indien we een beter begrip hebben van de genen en mechanismen die een rol spelen bij de totstandkoming van een complexe ziekte zou deze kennis gebruikt kunnen worden voor het ontwikkelen van specifieke medicijnen en het opzetten van testen om de ziekte te voorkomen.
Het doel van het onderzoek beschreven in dit proefschrift was om een bijdrage te leveren aan het verkrijgen van een beter inzicht in deze genen en mechanismen. Het gebruik van een diermodel kan hierbij helpen. Een voorbeeld van een diermodel voor complexe ziekten is het apomorfine-gevoelige (APO-SUS) en apomorfine-ongevoelige (APO-UNSUS) rattenmodel. Zowel genetische- alsook omgevingsfactoren spelen een rol bij de totstandkoming van het fenotype van de APO-SUS/-UNSUS ratten. Aangezien de APO-SUS ratten bovendien een aantal kenmerken vertonen die ook worden aangetroffen bij mensen met een complexe ziekte, kan het APO-SUS/-UNSUS rattenmodel bijdragen aan de zoektocht naar de genetische achtergrond van deze ziekten. In deel A van het proefschrift bestuderen we het APO-SUS/-UNSUS rattenmodel en vervolgens gebruiken we deze resultaten om de situatie bij de mens te onderzoeken (deel B).
In hoofdstuk A1 werd met behulp van microarray analyse (het vergelijken van mRNA expressieniveau's) van de hippocampus van APO-SUS en –UNSUS ratten gevonden dat, vergeleken met APO-UNSUS ratten, APO-SUS ratten een verlaagde hoeveelheid *Aph-1b* mRNA hadden. Dit verschil in mRNA expressie werd zowel in

de originele APO-SUS en –UNSUS rattenlijnen als in de onafhankelijke replicatie van de lijnen (die tien jaar later was opgezet) waargenomen. Vervolgens toonde genomische analyse van het *Aph-1b* locus aan dat APO-SUS en –UNSUS ratten een verschillend aantal *Aph-1b* genkopieën bezitten; APO-SUS ratten hebben één of twee *Aph-1b* genen, terwijl APO-UNSUS ratten drie kopieën dragen. De verlaagde hoeveelheid *Aph-1b* mRNA correspondeerde met het verminderde aantal *Aph-1b* genkopieën. Aph-1b is een component van het γ-secretase enzymcomplex dat betrokken is bij de klieving van een groep van transmembraaneiwitten (zoals het Alzheimereiwit APP, neureguline en Notch1-4) en bij vele (neuronale) ontwikkelingsbiologische signaaltransductieroutes. De expressieniveau's van de overige γ-secretase componenten (Aph-1a, preseniline 1 en 2, nicastrine and preseniline-enhancer 2) waren niet verschillend, terwijl γ-secretaseklieving van een aantal transmembraaneiwitten wel verschillend bleek te zijn tussen de APO-SUS en –UNSUS ratten. Daarnaast bleek het aantal *Aph-1b* genkopieën te corresponderen met een aantal gedragskenmerken (zoals de apomorfine gevoeligheid en de activiteit op het open veld).

De hoodstukken A2 en A3 beschrijven in meer detail de verstoorde *Aph-1b* mRNA expressieniveau's en γ-secretase klievingsactiviteit in het APO-SUS/-UNSUS rattenmodel. De mRNA expressieniveau's van *Aph-1b* en van de paralogen *Aph-1aS* and *-1aL* werden tijdens de ontwikkeling bestudeerd (beginnend op embryonale dag 13 en eindigend op postnatale dag 100). Hieruit bleek dat gedurende de gehele ontwikkeling het verschillende aantal *Aph-1b* genkopieën leidde tot een verschillende mate van *Aph-1b* mRNA-expressie, terwijl de *Aph-1a* mRNA expressieniveau's zowel pre- als postnataal vrijwel gelijk waren. Bovendien werden voor een aantal transmembraaneiwitten, te weten APP (en de twee familieleden APLP1 en APLP2), $p75^{NTR}$, ErbB4 en neureguline-2, weefselspecifieke veranderingen in γ-secretase klievingsactiviteit gevonden. We kunnen dus in feite concluderen dat de verlaagde *Aph-1b* mRNA expressieniveau's en de verlaagde γ-secretase klievingsactiviteit tijdens de gehele ontwikkeling van de APO-SUS ratten kan leiden tot verstoorde (neuronale) ontwikkelingsbiologische signaaltransductieroutes en daardoor kan resulteren in het complexe fenotype van de APO-SUS ratten. Experimenten gebaseerd op kruisingen, genetische selectie en daaropvolgende fenotypische analyses hebben echter aangetoond dat het verschil in *Aph-1b* genkopieën niet de enige verklaring is voor de fenotypische verschillen tussen de APO-SUS en –UNSUS ratten.

In de hoofdstukken A4 en A5 hebben we een aantal andere genetische variaties aangetoond die zowel in de originele als in de onafhankelijke replicatie van de APO-SUS/-UNSUS rattenlijnen voorkomen. We hebben acht grote en vijf kleine DNA variaties alsmede een epigenetische variatie (DNA methylatie) in het DNA van de APO-SUS en –UNSUS ratten geïdentificeerd. Samen met het verschil in het aantal

Aph-1b genkopieën, zou een deel van deze nieuwe variaties dus kunnen bijdragen aan het complexe APO-SUS fenotype.
Het doel van het onderzoek was verder om de resultaten verkregen met het APO-SUS/-UNSUS rattenmodel te gebruikem om mechanismen bij de mens te bestuderen. Daarom hebben we vervolgens associatiestudies bij de mens uitgevoerd om te onderzoeken of aantasting van de γ-secretaseklieving een rol speelt bij de totstandkoming van complexe humane ziekten. In hoofdstuk B1 hebben we een polymorfisme van een enkel nucleotide ("single-nucleotide polymorphism", SNP) in het humane *APH1B* gen beschreven (leidend tot het aminozuur leucine, L, in plaats van het evolutionair geconserveerde aminozuur fenylalanine, F, op positie 217 van het eiwit). Een 217L-bevattend γ-secretase bleek het transmembraaneiwit syndecan-3 minder goed te klieven. We kunnen dus veronderstellen dat de F217L SNP van belang is voor de functie van het enzym. Onze associatiestudies lieten een verband zien tussen deze SNP en vroegtijdige atherosclerose bij mannen (hoofdstuk B1), epileptische aanvallen (hoofdstuk B2), HIV-1 infectie (hoofdstuk B3) en darmkanker (hoofdstuk B4). Daarnaast werd het risico-allel vaker aangetroffen in patiënten met een bipolaire stoornis (manisch-depressieve stoornis), autisme, ADHD, dyslexie, reumatoïde artritis, coeliakie (glutenenteropathie), hoofd- en nekkanker, prostaatkanker en longkanker. Echter, deze laatste associaties bleken niet significant te zijn, waarschijnlijk vanwege het relatief kleine aantal patiënten in deze groepen en het geringe voorkomen van de F217L SNP bij de mens (hoofdstuk B4). Uit bovenstaande resultaten kunnen we concluderen dat het γ-secretase-enzym een rol kan spelen bij de totstandkoming van een aantal complexe ziekten, waaraan dus een vergelijkbare biologische oorzaak ten grondslag kan liggen.
Samenvattend kan gesteld worden dat de resultaten van het onderzoek beschreven in dit proefschrift suggereren dat een verandering in γ-secretase klievingsactiviteit kan leiden tot een breed scala aan complexe ziekten.

Dankwoord
Curriculum vitae
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Dankwoord

Jawel, het is zover. De laatste bladzijdes van dit proefschrift kunnen geschreven worden; de berg is beklommen. Aan de voet van de berg begonnen op 1 april 2002, en nu, september 2008 kan ik terugblikken op een geweldige tocht met vele mooie, spannende en uitdagende momenten, maar natuurlijk ook, zoals bij elke tocht, met soms moeilijke en frustrerende momenten. Natuurlijk kon deze tocht alleen maar slagen door de tomeloze inzet van velen, die ik graag middels dit dankwoord zou willen bedanken.

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Karen

Curriculum vitae

Karen Miriam Johanna van Loo werd op 11 augustus 1979 geboren te Heerlen. Na het behalen van haar VWO diploma aan het Eijkhagencollege te Landgraaf begon ze in 1997 met de studie biologie aan de Radboud Universiteit Nijmegen (toen nog Katholieke Universiteit Nijmegen geheten). Ze koos voor de richting medische biologie, met afstudeerstages op de afdeling Moleculaire Dierfysiologie (Radboud Universiteit Nijmegen) onder leiding van prof. dr. Gerard Martens en dr. Marcel Coolen, en de afdeling Celfysiologie (UMC St Radboud) onder leiding van dr. Peter Deen en dr. Paul Savelkoul. In het voorjaar van 2002 werd het doctoraaldiploma behaald. Aansluitend begon ze als assistent in opleiding (AIO) aan haar promotie-onderzoek op de afdeling Moleculaire Dierfysiologie (Radboud Universiteit Nijmegen), onder leiding van prof. dr. Gerard Martens. Na afloop van vier jaar onderzoek werd een gedeelte van de onderzoekslijn gedurende een jaar voortgezet binnen een samenwerkingsproject van het farmaceutisch bedrijf Synthon BV te Nijmegen en de afdeling Moleculaire Dierfysiologie. Sinds maart 2007 is ze werkzaam als onderzoeker (postdoc) op het TI-Pharma project T5-209 “Novel susceptibility pathways and drug targets for psychosis”, een samenwerkingsproject van de afdeling Moleculaire Dierfysiologie (Radboud Universiteit Nijmegen), de afdeling Medische Farmacologie (Universiteit Leiden) en het farmaceutisch bedrijf H. Lundbeck A/S te Valby in Denemarken. Vanaf 1 februari 2009 zal ze werkzaam zijn als postdoc bij prof. dr. Albert Becker en dr. Susanne Schoch op de afdeling Neuropathologie aan de Universiteit van Bonn (Duitsland), waar ze de moleculaire achtergrond van epilepsie zal gaan bestuderen.

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