Molecular understanding of a rat model with schizophrenia-related features

Gene-dosage imbalance of the gamma-secretase component Aph-1b in APO-SUS and -UNSUS rats

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Chapter 1

GENERAL INTRODUCTION

General introduction

Schizophrenia is a devastating psychiatric disorder affecting about 1% of the world's population throughout countries, cultural groups, and sexes (Jablensky, 1997). In 1902, it was Kraeplin who first described the disease as *dementia praecox*, a term used to describe individuals who exhibited symptoms that involved severe mental deterioration from an early age. Later, Bleuler used the term *schizophrenia* to illustrate the theoretical rupture between thought, emotion, and behaviour (Bleuler, 1950). Today, schizophrenia is described as a psychiatric disorder characterised by abnormal mental functions and disturbed behaviour, mostly appearing after puberty as a diverse mixture of positive and negative symptoms, and cognitive impairment (Liddle, 1987). Positive symptoms involve loss of contact with reality, including paranoia, false beliefs (delusions), perceptual experiences not shared by others (hallucinations), or bizarre behaviours. Negative symptoms refer to diminished or absent basic emotional and behavioural processes, such as social withdrawal, reduced initiative, blunted affect, anhedonia (lack of pleasure), and reduced speech. The cognitive impairments in schizophrenia include attention deficits, reduced learning and memory, and executive dysfunctions (for example abstract thinking or problem solving) (Mueser and McGurk, 2004).

In addition, schizophrenic patients have shown an increased risk of alcohol and drug problems (Regier et al., 1990), infectious diseases (such as hepatitis C and HIV infection) (Rosenberg et al., 2001), violent victimization (Walsh et al., 2003) and post-traumatic stress disorder (Mueser et al., 2002), housing instability and homelessness (Susser et al., 1989), and negative emotions, such as anxiety (Huppert and Smith, 2001), depression (Addington et al., 1998), and hostility (Bartels et al., 1991). These risks cause among patients an increased mortality risk of about 10% due to suicide, accidents, and illnesses such as respiratory and cardiovascular diseases (Brown, 1997; Inskip et al., 1998). Surprising is the observation that although schizophrenic patients are often heavy smokers, they show a reduced risk of developing lung cancer (de Leon et al., 1995; Harris, 1988).

Aetiology

To date, the understanding of schizophrenia remains very rudimentary. Many attempts have been made to find out more on the cause of this disease. Groups from all over the world have been performing studies at the molecular, cellular and anatomical level in search for definitive genes or pathogenic molecular mechanisms, but thus far little progress is being made. The complex, diverse clinical phenotype and course of the disease tamper research progress, since they often result in inconsistent, or even contradictive findings. The efforts to identify the underlying mechanisms in schizophrenia can roughly be divided into three general lines of research. The first line of analyses focuses on the examination of the mechanism of action of the drugs that alleviate or mimic the

symptoms of schizophrenia. As a second approach, the neuro-anatomical abnormalities in the brains of schizophrenic patients are examined. And thirdly, the genetics of susceptibility for schizophrenia is analysed.

Drugs and neurotransmitters

The first discovered beneficial drug to treat schizophrenia was chlorpromazine in the early 1950s. This drug, a so-called neuroleptic, turned out to block the D2 subtype of dopamine receptor (Creese et al., 1976; Seeman et al., 1976). Indeed, many of the traditional antipsychotic drugs act by blocking the dopamine receptors in the brain. Conversely, amphetamine, which increases the dopamine levels in the synaptic cleft, was found to induce schizophrenic symptoms in humans (Davis et al., 1991). Both types of drug effects led to the *dopamine hypothesis* of schizophrenia, which proposes that dysfunction in dopamine neurotransmission is the underlying cause of the symptoms of the disorder (Carlsson, 1988). More specifically, a hyperactivity of mesolimbic dopaminergic neurons is suggested to produce the positive symptoms of schizophrenia such as psychosis. Several research groups have reported that the dopaminergic system is indeed affected in schizophrenia (reviewed by Abi-Dargham, 2004; Laruelle et al., 2003). In this respect, an important study by Abi-Dargham et al. (2000) has clearly shown that increased synaptic dopamine concentrations are apparent in the striatum of drug-free schizophrenic patients.

A drug that induces both positive and negative symptoms is phencyclidine (PCP or angel dust). In addition, it causes cognitive deficits seen in many schizophrenic patients (Javitt and Zukin, 1991). PCP appears to act predominantly on glutamatergic *N*-methyl-D-aspartate (NMDA) receptors. Also two other NMDA receptor antagonists (ketamine and MK-801) produce schizophrenic symptoms, and antipsychotics (i.e. dopamine receptor antagonists) can reduce PCP-induced behaviour. This has led to the hypothesis that hypofunctioning of the NMDA receptors causes schizophrenia (Coyle, 1996; Olney and Farber, 1995). One aspect of the glutamate model that is still poorly understood is the observation of differential effects of short- and long-term administration of NMDA antagonists. Both regimens, however, can induce profound behavioural symptoms in rats and primates similar to those observed in schizophrenia in humans (reviewed by Jentsch and Roth, 1999).

Also the serotoninergic (5-HT) system has been frequently implicated in schizophrenia, based on the effects of drugs like lysergic acid diethylamide (LSD) and mescaline (Costall and Naylor, 1995). Although these drugs cause psychedelic hallucinations, there is very little evidence of a primary dysfunction of the serotonergic systems in schizophrenia. Also, repeated administration of LSD leads to behavioural tolerance, which clearly differs from the situation in schizophrenia (Braff and Geyer, 1980).

Neuroanatomical abnormalities

Brain abnormalities in schizophrenic patients have been extensively studied over the past century. Unfortunately, many of these studies failed, because drug treatments and

dietary abnormalities of schizophrenic may themselves have caused alterations in brain anatomy. The recent development of neuroimaging techniques such as magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), and positron emission tomography (PET) has given investigators a new set of tools that permit *in vivo* study of brain abnormalities. Consistent findings in the brains of schizophrenic patients are the increased ventricular size, and prominent alterations in the prefrontal cortex and hippocampus, whereby the ventricular size aberrations are already present at the onset of the disease symptoms (Weinberger et al., 1979). However, a significant overlap is found between the ventricular sizes in subjects with schizophrenia and controls, making this an ineffective anatomical diagnostic marker. The prefrontal cortex and hippocampus are two brain areas involved in emotional regulation and cognitive functions suggesting that psychotic symptoms observed in schizophrenia originate in these regions (Stevens, 1973; Torrey and Peterson, 1974). The prefrontal cortex, one of the projectory regions of the dopaminergic pathway, has been associated with schizophrenia, based on clinical, neuropsychological and neuroimaging findings (reviewed by Knable and Weinberger, 1997). The hippocampus is implicated due to a reduced size, shape and architecture, alterations in metabolic activity, and neurochemistry in schizophrenic patients (reviewed by Harrison, 2004). Still, these observations do not clearly indicate a general dysfunction of these brain structures, but rather imply subtle morphological, architectural and cellular changes affecting the neuronal connectivity and pathways throughout the brain.

Genetic factors

Genetic studies on schizophrenia patients and their relatives have shown that the risk of developing the illness is progressively greater in relatives who are more genetically related to the person with schizophrenia (Figure 1). The analyses of monozygotic twins, who have 100% identical genomes, revealed that genetic factors appear to be important in the development of schizophrenia (a concordance rate of 48%), with heritability estimates of approximately 80-85% (Cardno and Gottesman, 2000; Sullivan et al., 2003). The family studies also show that simple major gene effects are unlikely. More likely, multiple susceptibility genes exist that act in concert. This makes schizophrenia a complex genetic disorder, not caused by a single gene and not showing simple Mendelian inheritance. The results of the monozygotic twin studies also demonstrated that genetics are not sufficient to explain the entire pattern of occurrence (Cardno et al., 1999).

Environmental factors

Next to the genetic component of schizophrenia, it has become apparent that environmental factors contribute to the risk of developing the disease. Numerous studies have reported an increased occurrence of stressful events during the pre- and perinatal periods in schizophrenic patients (reviewed by McDonald and Murray, 2000). Examples of such events are obstetric complications, prenatal exposure to infection, external toxins, malnutrition or maternal stress during pregnancy (Brown et al., 2001; Jones et al., 1998; Susser and Lin, 1992; van Os et al., 2000). Stressors occurring later in life can trigger the clinical manifestation of schizophrenia. It has been described that

up to 46% of patients underwent some stressful life event in the three months preceding the first occurrence of schizophrenic symptoms (Norman and Malla, 1993; Ventura et al., 1989).

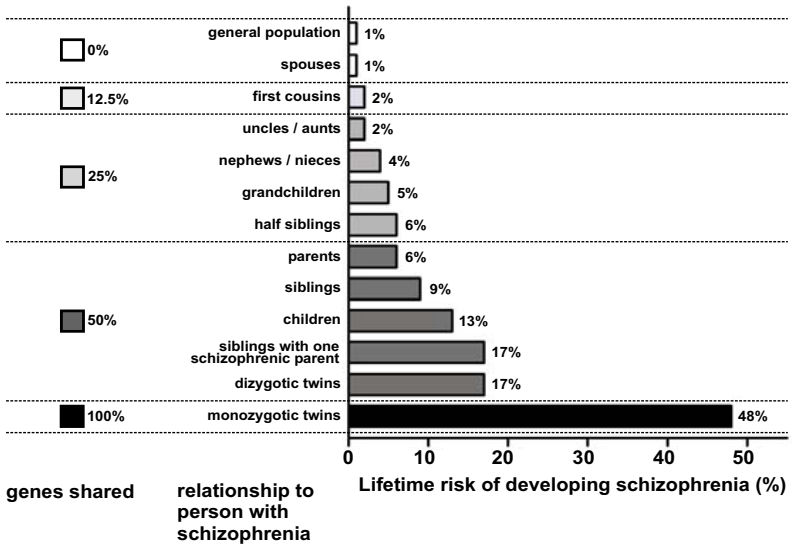

Figure 1. Genetic component to schizophrenia

Genetically related individuals show an increased risk of developing schizophrenia during their lives. Nevertheless, individuals with identical genomes (monozygotic twins) show a concordance rate of only 48%, indicating schizophrenia is not entirely a genomic disease. Adapted from Gottesman (1991).

Together, this forms the hypothesis that in schizophrenic patients a genetic susceptibility, together with stressful early-life events, results in neurodevelopmental impairment and affects maturation of the brain, manifesting in disease symptoms during or after puberty and mostly persisting throughout the patient's lifetime. This developmental aspect together with the long time interval until the onset of the first schizophrenic symptoms makes causal research very difficult. In this respect, prospective studies of (future) patients would be most informative, but on the other hand extremely expensive and time consuming due to the large cohorts to be studied over a long period of time.

Approaches

With the completion of the human genome sequencing project by the International Human Genome Sequencing Consortium in April 2003 a new era has begun enabling researchers to identify many new genes. Thus far, consortium researchers have confirmed the existence of 19,599 protein-coding genes in the human genome and identified another 2,188 DNA segments that are predicted to be protein-coding genes (2004). However, it could be years before a truly reliable gene count can be assessed. Moreover, to date the function of many of these genes is still unknown.

In the hunt for finding genes that may cause schizophrenia, the human genome sequencing data could be used in so-called genome-wide linkage analyses. To date, numerous of such genome-wide scans of schizophrenics have together implicated much of the genome, although only a couple of regions reached accepted levels of statistical significance (Owen et al., 2004). Unfortunately, each of these regions implicated have been refuted in other studies by not revealing any linkage with the disease (reviewed by Harrison and Weinberger, 2005). Due to this inconsistency the genome-wide linkage analyses approach has still not resulted in the discovery of a causative genetic region. The ultimate validation of a linkage analyses will come from the discovery of a gene or genes within a linked region, including functional information.

One other way of exploiting the public genome databases in the search for the genetic component of schizophrenia, is the testing for allelic variants of candidate genes, usually single-nucleotide polymorphisms (SNPs), within or adjacent to a gene. The identification of an association of a SNP with schizophrenics compared to a suitable comparison group, could mean that the SNP is either causative or in linkage disequilibrium (LD). Also in this case, the identification of susceptibility genes for schizophrenia will not come from mere statistical analyses, but will require biological evidence that the genetic risk variant has impact on the pathogenesis of the disease (Page et al., 2003).

Such alterations in genetic build-up may ultimately result in changes in gene expression, as measured by altered mRNA or protein levels. In addition, factors other than genetic mutations could also affect expression levels. Therefore, screening for differences at the RNA or protein level could be most informative in the search for susceptibility genes for schizophrenia. More recently, a new level has been added to the regulation of expression, termed epigenetics (Holliday, 1987). These epigenetic factors (heritable factors without sequence variation, such as DNA methylation, histone acetylation and chromatin structure) are able to regulate gene activity and are also believed to be important in schizophrenia (Jaenisch and Bird, 2003; Petronis, 2004; Robertson and Wolffe, 2000).

Expression analyses

The complexity of schizophrenia suggests that high throughput screening techniques, such as microarrays, may be well suited for identifying transcripts that are dysregulated in the disease state. High-density DNA arrays make it possible to monitor the expression levels of thousands of genes at a time. Other screening techniques such as differential hybridisation, subtractive library construction, representational difference analysis (RDA), suppression subtractive hybridisation (SSH), differential display (Liang and Pardee, 1992), conventional cDNA array hybridisation, serial analysis of gene expression (SAGE) (Velculescu et al., 1995), and (real-time) RT-PCR all have serious limitations for large genome-scale expression studies, as they are expensive, technically difficult and very labour intensive compared to the DNA microarray approach.

The two most widely used types of arrays to evaluate differential gene expression are the cDNA and oligonucleotide arrays, such as those produced by Affymetrix. These oligonucleotide arrays are synthesised *in situ* and consist of multiple, yet distinct, short

nucleotide stretches (e.g. 25-mers) of individual genes. Perfectly matching oligos are aligned adjacent to a control probe containing a single-base mismatch to correct for non-specific hybridisation. Alternatively, cDNA arrays consist of longer clone fragments (usually between 300 and 2000 bases) that are spotted on the array. This approach causes a greater flexibility and lower relative production cost, but it less suitable to detect specific splice variants or gene family members (Figure 2).

In the analyses of expression profiles (either at the RNA or protein level) several difficulties will be encountered. Firstly, the availability of post-mortem brain tissue is essential for expression studies. The cause of death, the age of the deceased, and interval between death and tissue storage or fixation are all complicating factors, which makes it almost impossible to find the perfect controls for proper expression analyses. Secondly, the (medical) history is likely to interfere with the experimental outcome. Each individual has a unique environment in which he or she grows up (family studies compensate in part for this). Furthermore, if a patient is on medication, the timing and dosage of the drugs can have a severe effect on the expression of a gene. Thirdly, since schizophrenia is a neurodevelopmental disorder with causal implications for early-environmental factors and a long time interval until the onset of symptoms, it will be quite a challenge to find causal expression differences between post-mortem samples from patients and controls.

Animal models

Animal models seem to be the perfect way of circumventing the difficulties encountered when using patient material for expression profiling analyses. Unfortunately, the modelling of human psychiatric disorders like schizophrenia in animals meets difficulties, since behavioural symptoms involving human communication and language (such as delusions or hallucinations) are difficult to simulate in animals. In addition, there is concern that especially in rodents some brain areas are not developed to the level necessary to express psychotic behaviours. Nonetheless, there are behaviours in animals that mimic certain symptoms of schizophrenia, such as locomotor hyperactivity, stereotypies, cognitive impairments, disruption of latent inhibition or prepulse inhibition, and electrophysiological abnormalities (Gainetdinov et al., 2001; Lipska and Weinberger, 2000). Especially the use of animal models in exploring the neurodevelopmental hypothesis in schizophrenia will be of vital importance. One of the major advantages of the use of animal models in studying schizophrenic symptoms is the ability to condition and control their environment. But also embryonic to adult studies can be performed much more easily and less expensive in animals compared to humans.

In general, the validity of animal models can be divided into three main categories: predictive validity, face validity and construct validity (Cools and Ellenbroek, 2002; Ellenbroek and Cools, 1990). In the first category of animal models - predictive validity is at the empirical level - the effects of substances or treatments on the animals are used to predict the situation in human. These models do not necessarily have any resemblance to the disease phenotype. Secondly, animal models with face validity, or symptom

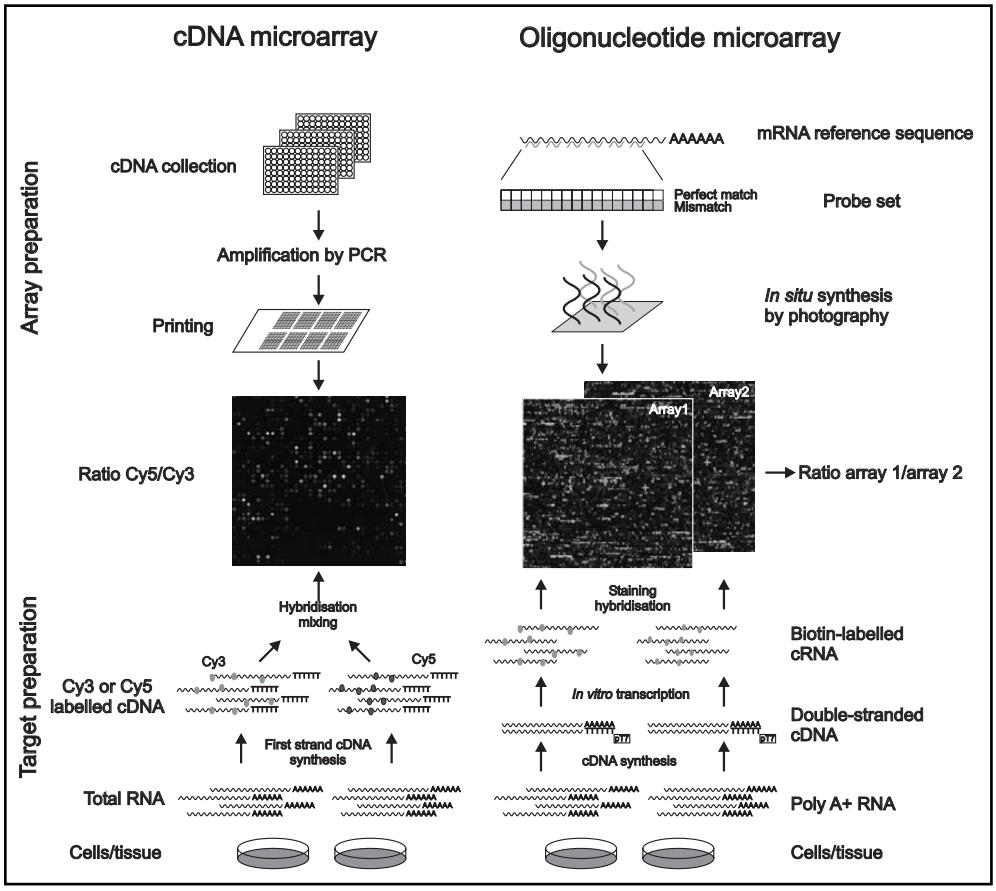

Figure 2. Comparison of cDNA and oligonucleotide array preparation and analysis

The cDNA microarrays are prepared by amplification of inserts from cDNA collections or libraries and subsequent printing of the PCR products on glass slides. The high-density oligonucleotide microarrays are prepared by selection of sequences of 16-20 short oligonucleotides (typically 25-mers) from the mRNA reference sequence of each gene and these oligonucleotides are synthesised *in situ* via a light-directed method, whereby high-density probe arrays containing over 300,000 individual elements are generated. The target preparation is also different for both types of microarrays. For the cDNA microarray, RNA from two controller and tester is used to synthesise single-stranded cDNA in the presence of nucleotides labelled with two different fluorescent dyes (for example, Cy3 and Cy5). Both samples are mixed and hybridised to the array surface, resulting in competitive binding of differentially labelled cDNAs to the corresponding array elements. Subsequent fluorescence scanning of the array provides relative signal intensities and ratios of mRNA abundance for the genes represented on the array. The target preparation for the oligonucleotide microarays occurs through polyA$^+$ RNA isolation from controller and tester, followed by double-stranded cDNA synthesis and concurrent incorporation of the T7 DNA polymerase transcriptional start site. During *in vitro* transcription, biotin-labelled nucleotides are incorporated into the synthesised cRNA molecules, and each target sample is hybridised to a separate probe array. After staining with a fluorescent dye coupled to streptavidin and scanning of the array, signal intensities of different arrays are used to calculate relative mRNA abundance for the genes represented on the array (adapted from Schulze and Downward, 2001).

validity, have a perceived resemblance with the human disease, but without a similar cause (Willner, 1986). Face validity models can be generated by behavioural selection of animals, they can be pharmacologically induced, or they can be experimentally induced by manipulations such as brain lesions. Examples of such face validity models for schizophrenia are given below.

Animal models based on behavioural selection

One test to show the information processing deficit in the brains of schizophrenic patients is the pre-pulse inhibition of the acoustic startle response (PPI). The PPI refers to the ability of a weak pre-stimulus to transiently inhibit the response to a closely following strong sensory stimulus, and is found to be deficient in schizophrenia (reviewed by Braff et al., 2001). Performing PPI analysis among rat lines and strains have revealed marked differences (Ellenbroek et al., 1995; Glowa and Hansen, 1994; Markou et al., 1994). The division of rats in groups, based on their differences in PPI and subsequent analyses of the possible cause for their discrepancies, may also yield useful information for understanding certain aspects of schizophrenia.

Pharmacologically induced animal models

As mentioned earlier, the psychotic effects of psychomimetric drugs, such as amphetamine and phencyclidine (PCP), cause in healthy humans symptoms comparable to the positive symptoms in schizophrenia, and PCP also induces negative symptoms. The pharmacological animal models are based on the observations that such drugs will also evoke schizophrenia-like symptoms in animals. In rats, for example, PCP induces hyperactivity, stereotypy, ataxia and impaired social behaviour which can be ameliorated by clinically effective neuroleptic drugs (reviewed by Morris et al., 2005), and it disrupts PPI (Geyer et al., 2001). Such animal models may aid especially in testing potential novel treatments prior to clinical studies in patients.

Brain lesion animal models

Animal models for schizophrenia using the concept of brain lesions have shown that especially neonatal lesions appear to mimic schizophrenic symptoms. Monkeys with a neonatally lesioned hippocampus show impairment in relational learning, reduced social behaviour and increased locomotor stereotypy (Bachevalier et al., 1999). Additionally, neonatal disconnections of the hippocampus or amygdala in rats have also been reported to function as models for schizophrenic symptoms (Flores et al., 2005; Lipska, 2004; Wolterink et al., 2001). Also chemical lesions such as the injection of 6-OHDA within the nucleus accumbens of neonatal rats resulted in an impaired prepulse inhibition and were suggested to represent an animal model for schizophrenia as well (Bubser and Koch, 1994). Unfortunately, neonatal lesioning of brain regions will aid in understanding what brain structures are involved in the disease symptoms, but it most likely will not result in finding causative genetic defects.

The third main category of animal models shows construct validity, and originates from the theoretical level. In these models, the actual cause of the human disorder is mimicked. As a result, large theoretical and practical similarities in cause, aetiology and symptoms can exist between animal model and human disorder (Ellenbroek and Cools, 1990; Willner, 1986). As examples for models with construct validity, genetically modified animal models and the innovative APO-SUS/-UNSUS rat model are described below.

Genetic animal models

Completion of the genome sequencing of the mouse and rat in 2002 and 2004, respectively, has enabled researchers to further exploit these rodents as animal models to study (characteristics of) complex disorders at the genetic level. Genetic animal models for schizophrenia have been focusing predominantly on knockout or transgenic mice with a single genetic mutation. For this approach, the mouse has been a favourite tool of geneticists, due to the relatively easy technical procedures for manipulating individual genes. One of the major drawbacks of the use of mice in studying complex psychiatric traits is that mice have only limited use in complex behavioural research compared to rats. This would favour rats above mice in the use of studying schizophrenic behaviour. Unfortunately, the specific manipulation of rat genes has appeared to be much more difficult. Only via recently described techniques, such as the ENU mutagenesis, random mutations can be incorporated in the genome of laboratory rats and via genetic screens specific mutations could be further analysed (Smits et al., 2004). But this approach still has to prove its usefulness. Furthermore, these knockout or mutational approaches in mice and rat has provided useful models for Mendelian, but not for complex diseases.

APO-SUS and -UNSUS rat model

For the study of genetic background of complex diseases (i.e. with a presumed multigenic cause), a different approach will likely be most effective. So-called heuristic (i.e. "serving to discover" or innovative) models may help to uncover mechanisms of schizophrenia-like phenomena. A good heuristic model will show more schizophrenia-like abnormalities besides the feature that is directly manipulated. One example of such a heuristic animal model is the APO-SUS/-UNSUS rat model.

In the past, Cools et al. (1990) generated a rat model, not focusing primarily on a genetic deficit, but rather on the behavioural phenotype. They used the approach to pharmacogenetically select rats from an outbred Wistar population with either a high or a low susceptibility of the dopamine agonist apomorphine (referred to as APO-SUS and APO-UNSUS rats, respectively). The apomorphine susceptibility of the Wistar rats as measured by their gnawing behaviour, revealed a bimodal distribution within the population. Both the vigorously gnawing and non-gnawing rats were used to set up outbred lines, preventing brother-sister pairing. Both rat lines thus represent two extremes of a population of Wistar rats. Extensive phenotyping of the APO-SUS and -UNSUS rats has revealed differences in many aspects of behaviour, neuroanatomy,

and their neurochemical, endocrine and immune systems (Cools and Gingras, 1998; Ellenbroek and Cools, 2002). Interestingly, APO-SUS display a variety of features closely resembling those observed in patients suffering from schizophrenia, including increased sensitivity to dopamimetic drugs (Ellenbroek et al., 2000; Muller-Spahn et al., 1998), increased level of *tyrosine hydroxylase* mRNA in the substantia nigra (Rots et al., 1996; Toru et al., 1984), decrease in prepulse inhibition and latent inhibition (Baruch et al., 1988; Braff and Geyer, 1990; Ellenbroek et al., 1995), a retarded development (Degen et al., 2005; Wahlbeck et al., 2001), increased hypothalamus-pituitary-adrenal (HPA) axis response to stress (Lammers et al., 1995; Rots et al., 1995), relative dominance of TH2 cells (Kavelaars et al., 1997; Muller et al., 1999), increased occurrence of periodontitis (Breivik et al., 2000; Tang et al., 2004), reduced sensitivity to develop arthritis (van de Langerijt et al., 1994; Vinogradov et al., 1991) and lower risk of developing (lung) cancer (Mortensen, 1994; Teunis et al., 2002) (Table 1).

Table 1. Phenotypic similarities between APO-SUS rats and schizophrenic patients (with internal reference list)

	Observation	
Characteristics	**APO-SUS**	**schizophrenia**
Information processing		
prepulse inhibition	reduced[1]	reduced[2]
latent inhibition	reduced[1]	reduced[3]
Dopaminergic pathway		
TH mRNA in SNPC	increased[4]	increased[5]
D2-binding in dorsal striatum	increased[4]	increased[6]
DRD1 mRNA level in lateral caudate putamen	increased[4]	unknown
metabolic activity in globus pallidus	increased[7]	increased[8]
apomorphine susceptibility	increased[9,10]	increased[11]
susceptibility to dopamimetric drugs	increased[9]	increased[11]
Endocrine system		
HPA-axis	hyper-reactive[4,12,13]	hyper-reactive[14,15]
Immune system		
TH2/TH1 cell count	high[16]	high[17]
NK cell activity in spleen	reduced[18]	inconclusive[19,20]
sensitivity for rheumatoid arthritis	reduced[21]	reduced[22]
Occurrence of periodontitis	increased[23]	increased[24]
Lung cancer carcinoma metastasis	lower rate[25]	lower rate[26]

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In addition, it has been shown that both genetic and environmental factors play an important role in the severity of the observed phenotype in the rats. Crossbreeding experiments have proven the involvement of genetic factors (Ellenbroek et al., 2000). Furthermore, the environmental contribution to the phenotype became apparent from the observation that either the application or the lack of stress during early life causes differences in expression of the apomorphine susceptibility in the adult rats. For example, when APO-SUS pups are reared from birth on by APO-UNSUS mothers their susceptibility is significantly reduced, whereas such cross-fostering 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) (Figure 3). Interestingly, the APO-SUS and -UNSUS rat lines have been generated twice with a ten year interval and many of the phenotypic observations have been found in both the original and replicated lines (Cools et al., 1990; Ellenbroek et al., 2000).

Heuristic animal models, like the APO-SUS/-UNSUS rat model, could be valuable in the search for the genotype accompanying complex phenotypes. The recent completion of the sequencing of the human, rat and mouse genomes, and the development of powerful genome-wide screening techniques will almost certainly aid in the search for causative genes in complex diseases, like schizophrenia.

Scope and outline of this thesis

The aim of this study is to identify genes that underlie the phenotypic characteristics of the APO-SUS/-UNSUS rat model. Opposed to their phenotypic counterpart, the APO-UNSUS rats, APO-SUS rats display a complex phenotype reminiscent of schizophrenia. Schizophrenia is a chronic, severe, and disabling mental disorder of which the cause is still not known. Understanding the molecular mechanism underlying the APO-SUS/-UNSUS complex phenotype might aid in the finding the cause of schizophrenia in human.

Chapter 1 lays out the characteristics and aetiology of schizophrenia, and the current paths followed to search for the cause of this severe mental disorder. The need for heuristic animal models is also stressed out in order to further analyse the neurodevelopmental hypothesis of schizophrenia. Finally, the phenotypic characteristics of the APO-SUS/-UNSUS rat model are compared with those of schizophrenic patients.

Chapter 2 describes the quest for gene transcripts whose levels are affected in the hippocampus of APO-SUS compared to -UNSUS rats. In this search, we analysed the gene expression profiles at both the juvenile and adult stage using the microarray approach. The analysis revealed remarkably few affected transcript levels in the rat model. Upon validation of the microarray results by quantitative RT-PCR, the transcript with the largest difference turned out to be the result of a dissection artefact. Part of the choroid plexus, an epithelial cell layer directly surrounding the hippocampus, was co-dissected with hippocampal samples from a number of rats causing some of the observed

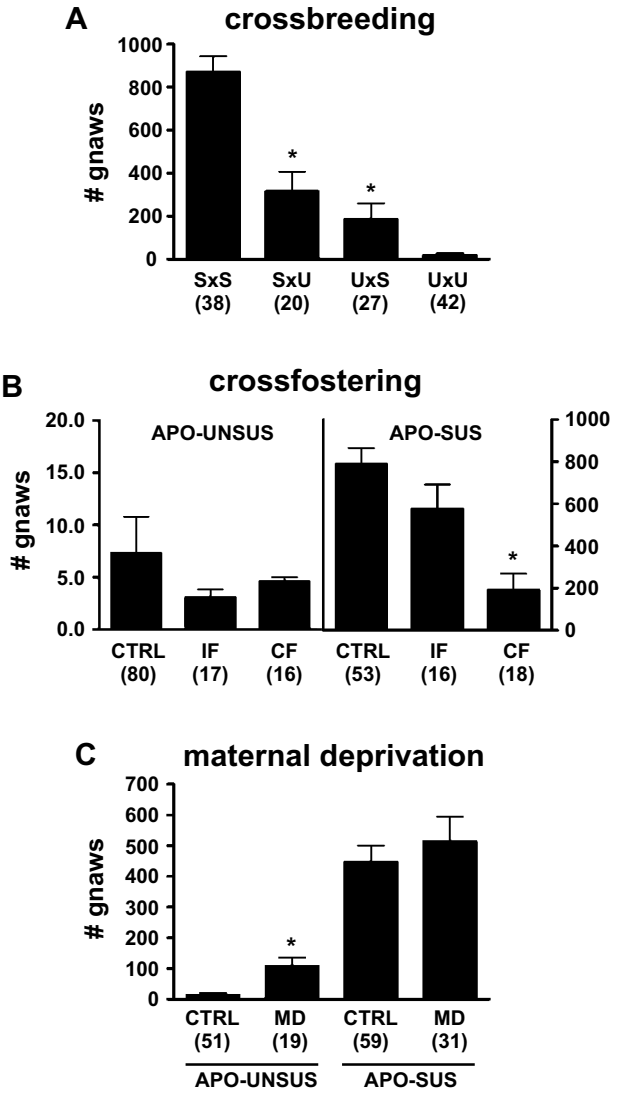

Figure 3. Effects of cross-breeding, cross-fostering and maternal deprivation on the apomorphine susceptibility of APO-SUS and -UNSUS rats

(A) Crossbreeding of APO-SUS (S) and APO-UNSUS (U) rats significantly changes the gnawing scores of the offspring (UxS and SxU). (B) Cross-fostering (CF) of APO-SUS pups by an APO-UNSUS mother significantly reduced the gnawing scores of these APO-SUS rats in adulthood. No significant effects were observed in APO-UNSUS rats or upon in-fostering (IF). (C) Maternal deprivation (MD) at PND9 significantly increased the gnawing scores of APO-UNSUS rats, whereas this treatment had no significant effect on APO-SUS rats. The number between brackets refers to the number of animals tested; *: $P < 0.05$ (adapted from Ellenbroek et al., 2000).

differential expression levels. In fact, after validation only one transcript, *Aph-1b*, was found to be consistently reduced in the APO-SUS compared to -UNSUS rats. These differences were found in multiple tissues, both in male and female rats, and in both the original and the replicated lines of the APO-SUS and -UNSUS rats. The possible causes for the differential expression of *Aph-1b* mRNA are discussed.

Chapter 3 reports the search for the cause of the reduced expression levels of *Aph-1b* mRNA in APO-SUS compared to -UNSUS rats. Detailed genomic analyses revealed differences in the genetic locus of *Aph-1b*. The APO-SUS rats harbour only one or two copies of *Aph-1b* (I/I or II/II rats, respectively), whereas APO-UNSUS rats contain three gene copies (III/III rats). The most likely cause of the alteration in gene-copy numbers is presented. Since Aph-1b is part of the γ-secretase enzyme complex that is involved in multiple (neuro)developmental signalling pathways, also other γ-secretase components have been tested for their expression levels in his rat model, followed by an analysis of the activity of the γ-secretase complex towards various substrates. Finally, behavioural tests are described testing whether a direct correlation exists between the genotype and the phenotypic characteristics.

Chapter 4 deals with a detailed developmental expression analysis of the mRNA levels of the *Aph-1* family members (*Aph-1b*, and its paralogues *Aph-1aS* and *-1aL*) in the rats with one, two or three *Aph-1b* copies. Also the consequences of the developmental dynamics in *Aph-1b* expression levels on the cleavage activity of the γ-secretase complex towards the amyloid-β precursor protein (APP) are presented. The effects of the altered γ-secretase activities during ontogenesis of the APO-SUS rats on the development of the complex neurodevelopmental phenotype are discussed.

In ***chapter 5***, the tissue distributions of the Aph-1 family members in both APO-SUS and -UNSUS rats are described. This analysis shows large differences in the mRNA levels of *Aph-1b* relative to *Aph-1a* between the tissues analysed. In both rat lines, the functional consequences of these differences are tested towards a number of γ-secretase substrates. Our findings are summarised in a model supporting the causative link between an affected γ-secretase component and the observed complex phenotype in the APO-SUS rats.

Chapter 6 describes the use of *Xenopus laevis* stable transgenesis for the functional analysis of the Aph-1 protein. The generation of frogs expressing a fluorescently labelled Aph-1 fusion protein specifically in the neurointermediate lobe of the pituitary is outlined, and a rationale for this approach is given. The consequences of the overexpression of this fusion protein are examined at the protein, cellular, physiological and organismal level.

Chapter 7 discusses the possible causes of the genomic rearrangement in the *Aph-1b* locus, as found in the APO-SUS/-UNSUS rat model. Interesting future experiments on rats with different *Aph-1b* gene copy numbers are suggested to further understand the influence of the *Aph-1b* gene-dosage imbalance on the complex phenotype. The data presented in this thesis, together with earlier observations in the rat lines, are put into a general model explaining the observed phenotype. Furthermore, the clinical implications of our results in the search for the cause of schizophrenia are discussed and suggestions

for translational research are presented, in which our data could be the starting point for a new direction in finding the cause of and a cure for schizophrenia.

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Chapter 2

GENE EXPRESSION PROFILING IN A RAT MODEL WITH SCHIZOPHRENIA-RELATED FEATURES

with
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Abstract

Wistar rats pharmacogenetically selected for a high susceptibility to the dopamine agonist apomorphine (APO-SUS rats) display many differences with their phenotypic counterpart (APO-UNSUS rats). These differences encompass a variety of pharmacological, biochemical, endocrinological, immunological as well as behavioural characteristics that are remarkably similar to those observed in schizophrenic patients. In the present study, the mRNA expression profiles were determined in the hippocampus and cerebellum of APO-SUS and -UNSUS rats at postnatal days 9 and 60 with oligonucleotide and cDNA microarrays, respectively. The results showed that ~0.1% of all genes tested were differentially expressed between the two rat lines, with the largest differences observed for *transthyretin* and *prostaglandin D synthase* mRNA. However, validation studies revealed that these two differences were due to a dissection artefact. The only reproducible alteration in transcript levels concerned the reduced amount in APO-SUS rats of the mRNA encoding Aph-1b, a component of the γ-secretase enzyme complex. This difference was found in all tissues examined, in both male and female rats, and in the original as well as the replicated lines of the APO-SUS and -UNSUS rats. Together, these results illustrate the importance of accurate tissue dissection and data validation of microarray experiments, and the complexity of analysing brain tissues. Most importantly, our studies suggest that the γ-secretase component Aph-1b is responsible for the phenotypic characteristics of the APO-SUS/-UNSUS rat model.

Introduction

Over the last decades, it has become more and more clear that the elucidation of the molecular genetic basis of psychiatric diseases is an extremely difficult task. The problem already starts with the heterogeneity in the disease profiles and the subjective clinical evaluation of a complex phenotype. A classical example of a complex disorder is schizophrenia, which shows a large variety of symptoms partially overlapping with diseases such as autism, bipolar disorder and major depression (Bearden et al., 2004; Stahlberg et al., 2004). Additional difficulties in studying human psychiatric disorders are factors that can bias the results, such as individual differences in environmental conditions to which the patients have been exposed (including the way the individuals have been raised), their medical history, and possible substance abuse. The utilisation of animal models could accelerate the pace of genetic research on psychiatric phenotypic characteristics, since such models can easily be phenotypically selected, bred, and conditioned. This is particularly important for studying psychiatric disorders because many of these disorders have a neurodevelopmental origin. An example of an animal model for a complex neurodevelopmental phenotype is the APO-SUS/-UNSUS rat model. Apomorphine-susceptible (APO-SUS) rats are pharmacogenetically selected Wistar rats with a vigorous gnawing response upon injection of the dopaminergic receptor agonist apomorphine. In contrast, their phenotypic counterpart the apomorphine-

unsusceptible (APO-UNSUS) rat line shows hardly any response to the drug (Cools et al., 1990; Ellenbroek and Cools, 2002). Next to this apomorphine susceptibility, the APO-SUS rat line displays many features of a complex neurodevelopmental disorder, including a reduced pre-pulse inhibition and latent inhibition, hyperactivity in an open field and in the elevated-plus maze, a hyper-reactive dopaminergic pathway, an increased stress response, a retarded development, a lower rate of metastasis in lung cancer, and enhanced vulnerability to drug abuse and a variety of other behavioural, neurochemical, endocrinological and immunological characteristics (Degen et al., 2005; Ellenbroek and Cools, 2002; Teunis et al., 2002; van der Kam et al., 2005). Cross-breeding, cross-fostering and maternal deprivation experiments have shown that both genetic and environmental factors play an important role in the development of the rat model (Ellenbroek et al., 2000). Interestingly, the APO-SUS/-UNSUS rat model was created twice with a ten-year interval and the replicated line displayed similar features as the original one (Ellenbroek et al., 2000). Here, we performed mRNA expression profiling using cDNA and oligonucleotide microarray analyses to search for genes differentially transcribed in the APO-SUS and -UNSUS rat model.

Materials and methods

Animals

Systemic administration of apomorphine (1.5 mg/kg s.c.) produced Wistar 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. The procedure for the generation of the APO-SUS/-UNSUS rat lines was performed twice over time with a ten year interval, resulting in an original APO-SUS and -UNSUS rat line and a replicated APO-SUS and -UNSUS rat line (Ellenbroek et al., 2000). For the present microarray studies, we used APO-SUS and -UNSUS rats of the 13th to the 18th generation of the replicated lines. For the validation studies, we also used APO-SUS and -UNSUS rats of the 28th generation of the original 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.

cDNA and oligonucleotide microarray experiments

For mRNA expression profiling, hippocampi and cerebelli were dissected from fresh brains of postnatal day (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 individual samples were pooled and used in the microarray analyses. 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 6,746 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. The cDNA microarray experiments were performed with biological duplicates. The PND 9 hippocampal and cerebellar 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. The internal controls for the reverse transcription and hybridisation processes indicated that the array samples were successfully labelled and hybridised to the arrays for both cDNA and oligonucleotide arrays.

Rat cDNA and oligonucleotide microarray specifications

The spotted NeuroGEM 2.02 cDNA microarray (Incyte Genomics, Inc.) contained 8,478 sequence-verified cDNAs with an average length of 1,000 bp (500-5,000 bp range). The cDNAs were derived from 18 different nervous tissues, including spinal cord, whole brain and cerebrum of Sprague Dawley rats and chemically bonded to the glass surface of the microarray. The array contained 6,747 unique elements of which 3,790 were annotated and 2,957 were not annotated in the Ensemble database of January 2000. The oligonucleotide array U34A or GeneChip (Affymetrix, Inc.) contained probes interrogating approximately 7,000 mRNA transcripts and approximately 1,000 EST clusters. Each sequence was represented by 16 perfectly matching probes of 25 nucleotides, along with 16 probes with a single-nucleotide mismatch for background subtraction.

Quantitative RT-PCR

For quantitative RT-PCR, first-strand cDNA was prepared from 2 µg of 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 15s at 94 °C, 30s at 60 °C and 1 min at 72 °C. β-*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 primers used are summarised in Table 1.

Table 1. Primer sequences used for quantitative RT-PCR validation

Included are Genbank numbers, amplicon sizes and positions of 5'-ends of primers relative to the start ATG; *): position from start of known sequence given in Appendix 1.

			amplicon	
primer name	**5'-sequence-3'**	**position**	**size**	**GenBank**
βactin-FW	5'-CGTGAAAAGATGACCCAGATCA-3'	346	89	V01217
βactin-RV	5'-AGAGGCATACAGGGACAACACA-3'	435		
TTR-FW	5'-CCTCGCTGGACTGATATTTGC-3'	30	78	X14876
TTR-RV	5'-ACTTTGACCATCAGAGGACACTTG-3'	108		
PGDS-FW	5'-CGGCCTCAACCTCACCTCTA-3'	225	162	M94134
PGDS-RV	5'-GCGTACTCATCGTAGTCGGTTTCT-3'	387		
Aph1b-FW	5'-GTGATTCTCCTCAGTTCTTCCTTAATTC-3'	448	223	XM_217185
Aph1b-RV	5'-GCCCATGAGCACCATGATTATAT-3'	671		
αATLP-FW	5'-ACAAGACTCTAAAGCCAGCAAAGA-3'	413	263	AY113703
αATLP-RV	5'-GAAGTACCTCATCCTTGTGTGCTT-3'	676		
EST1-FW	5-CCGGTAGGATCTGCCTCCCAAA-3'	9*)	233	*(Appendix 1)*
EST1-RV	5'-CACCATGCTGTGGACCTTACTGAG-3'	242*)		
EST2-FW	5'-GCCAAATGAAGAAAGAAGACACTGA-3'	94*)	125	*(Appendix 1)*
EST2-RV	5'-AATCCAAAATTCCAGCACAGACTT-3'	219*)		
EST3-FW	5'-AACTCAATGTTTATTCACCTACCCATG-3'	128*)	85	*(Appendix 1)*
EST3-RV	5'-AACTTAATTCGTCTGTCTACTCCTCTTACA-3'	213*)		
Aph1a-FW	5'-AGAGGAGACGGTACTGGGCTTT-3'	547	123	BC087081
Aph1a-RV	5'-ATGGAAACGGTGACTGCATAGA-3'	670		
Vesl2-FW	5'-ACAGTGGAAGCGGCAATTCT-3'	627	95	NM_053309
Vesl2-RV	5'-TGTTGATCTCACCGCACTGTTC-3'	722		
S100related-FW	5'-GAGTGCTCATGGAAAGGGAGTT-3'	95	94	J03627
S100related-RV	5'-CGGCACTGGTCCAGGTCTT-3'	189		
Histone H4-FW	5'-TGTCAAGCGTATCTCGGGTCTTAT-3'	129	142	X13554
Histone H4-RV	5'-CGCGTACACCACGTCCATAG-3'	271		
PI3KP85-FW	5'-TTAATCTCAGCGGAGTGGAGTGA-3'	877	104	NM_013005
PI3KP55-FW	5'-CAAGACAGATATAAACTGTGGCACAGA-3'	48	123	D64048
PI3KP50-FW	5'-GGTGGGATACTGGCAGTTCAAA-3'	59	116	U50412
PI3common-RV	5'-GACATATTGTTGTTCATGCTGTTGTT-3'	981/171 /175		
CyclinG-FW	5'-ACTACTGCCTTCCAATTTCTGCA-3'	451	106	X70871
CyclinG-RV	5'-TCAGTTGGGCTTCTAGTCTTTCAA-3'	557		
COMT-FW	5'-CCACTGGAAAGACCGCTACCT-3'	552	101	BC081850
COMT-RV	5'-CCGGGACGATGACGTTGT-3'	653		
MAP2-FW	5'-CGGATCAACCGACAACATCA-3'	5025	209	NM_013066
MAP2-RV	5'-GAGCATTGTCAAGTGAGCCAACT-3'	5234		

(continued on next page)

(Table I continued)

CAPS-FW	5'-CAGACGTAGCATCAAGCAAGGT-3'	4472	135	NM_013219
CAPS-RV	5'-ACATTTATAGTCTAGGGTTGTCATTTCG-3'	4607		
HERC1-FW	5'-GCTCACCTCTGATTTGATGTTGATAT-3'	15166	83	XM_236362
HERC1-RV	5'-GGCCACTGCTTATGTCAGCTTAT-3'	15249		
CA12-FW	5'-GACTCCAGCCTCACCAGTACAGA-3'	323	103	XM_343416
CA12-RV	5'-GCAAAGTGCTTCCCACTCACA-3'	426		
Rab8B-FW	5'-CAGAACGATAGAACTCGACGGAA-3'	141	92	U53475
Rab8B-RV	5'-AGTATGCTGTTGTAATTGTTCGGAAT-3'	233		

In situ hybridisation

TTR mRNA. For the synthesis of the *TTR* mRNA probe, first, RNA from total rat brain was isolated and transcribed into cDNA as described above. The cDNA was then amplified by PCR using the following primers: *TTR* (84-409) 5'-CAAGTGTCCTCTGATGGTCA and 5'-TGTAGGAGTACGGGCTGAGCAG. Following ligation of the PCR fragment into the pGEM-T Easy Vector (Promega) and sequence analysis using the Big Dye Ready Reaction system (Perkin Elmer), the construct was linearised with *Sal*I or *Nco*I for the generation of 11-UTP digoxygenin (DIG)-labelled antisense or sense RNA probes as run-off transcripts using T7 or Sp6 RNA polymerase, respectively. For tissue preparation, rats were deeply anaesthetised by intraperitoneal administration of sodium pentobarbital (60 mg/kg), and transcardially perfused with 50 ml 0.9% NaCl solution, followed by 300 ml Bouin's fixative (71% picrinic acid, 24% formaldehyde, 5% glacial acetic acid). Brains were removed, postfixed in the same fixative for 24 h at 4°C, dehydrated in a graded series of ethanol and xylene and embedded in paraffin. Coronal sections of 10 µm were mounted on poly-L-lysin coated slides and dried for 16 h at 37°C. Sections were deparaffinated in xylene and rehydrated in a graded series of ethanol. Tissue penetration was enhanced by incubation in 0.1% pepsin in 0.2 M HCl for 15 min at 37°C, followed by a post-fixation in 2% formaldehyde in PBS for 5 min, and incubation in 1% $NH_2OH.HCl$ for 15 min. Sections were rinsed in RNase-free water, dehydrated in ethanol and air-dried. Hybridisation was performed for 16 h at 50°C in hybridisation buffer (10% sodium dextran sulphate, 50% formamide, 4x SSC, 1x Denhardt's and 200 µg/ml yeast tRNA; 1xSSC: 0.15 M NaCl and 15 mM sodium citrate) with 3 ng/ml antisense DIG-labelled *TTR* mRNA probe. After stringency washes in 2x SSC, 1x SSC, 0.5x SSC for 30 min and 0.1x SSC for 30 min at 37°C, sections were rinsed in buffer 1 (100 mM Tris pH7.5, 150 mM NaCl) for 5 min, blocked in 1% BSA and 2% normal goat serum in buffer 1 for 30 min, and incubated in alkaline phosphatase-conjugated sheep-anti-DIG Fab fragments (1:500, Roche) in blocking solution of 16 h at 4°C. After three washes of 5 min in buffer 1 and one wash of 5 min in AP-buffer (100 mM Tris pH9.5, 100 mM NaCl, 50 mM $MgCl_2$), sections were stained in 350 µg/ml 4-nitro blue tetrazolium chloride and 175 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche) in AP-buffer until colour development was sufficient in case of the anti-sense probe. Signal specificity was checked by in parallel hybridisation of slides with the sense *TTR* mRNA probe.

Aph-1b mRNA. For the synthesis of the *Aph-1b* mRNA probe, first, RNA from total rat brain was isolated and transcribed into cDNA as described above. A direct template for antisense RNA probe synthesis was then amplified by PCR using the following primers: *Aph1b*-FW: 5'-AGACAGCACCTTCGATGCGA-3' and T7-*Aph1b*-RV: 5'-TAATACGACTCACTATAGGGAGACACTTATTCTTCTCACAGCCGTCA-3', with the T7 promoter sequence underlined. For the sense probe the primers T7-*Aph1b*-FW: 5'-TAATACGACTCACTATAGGGAGAAGACAGCACCTTCGATGCGA-3' and *Aph1b*-RV: 5'-CACTTATTCTTCTCACAGCCGTCA-3' were used. The PCR products were gel purified and 1 µg of template was used to generate 11-UTP digoxygenin (DIG)-labelled antisense or sense RNA probes using T7 RNA polymerase. For tissue preparation, rats were decapitated, brains were snap-frozen in isopentane on dry-ice and 16 µm cryo-sections were mounted on poly-L-lysine coated object slides. Sections were fixed in 4% paraformaldehyde in PBS for 10 min, washed and treated with 0.1% pepsin in 0.2 M HCl for 5 min at 37°C. After washing in PBS, hybridisation was performed for 16 h at 50°C in hybridisation buffer (10% sodium dextran sulphate, 50% formamide, 4x SSC, 1x Denhardt's and 200 µg/ml yeast tRNA; 1xSSC: 0.15 M NaCl and 15 mM sodium citrate) with 160 ng/ml DIG-labelled antisense *Aph-1b* mRNA probe. After stringency washes in 5x SSC for 5 min and 0.2x SSC for 2 hours at 65°C, sections were rinsed in buffer 1 (100 mM Tris pH7.5, 150 mM NaCl) for 5 min, blocked in 1% BSA and 2% normal goat serum in buffer 1 for 30 min, and incubated in alkaline phosphatase-conjugated sheep-anti-DIG Fab fragments (1:500, Roche) in blocking solution of 16 h at 4°C. After three washes of 5 min in buffer 1 and one wash of 5 min in AP-buffer (100 mM Tris pH9.5, 100 mM NaCl, 50 mM $MgCl_2$), sections were stained in 350 µg/ml 4-nitro blue tetrazolium chloride and 175 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Roche) in AP-buffer until colour development was sufficient in case of the anti-sense probe. Signal specificity was checked by in parallel hybridisation of slides with the sense *Aph-1b* mRNA probe.

Statistics

For the selection of transcripts from the cDNA microarray experiments for follow-up analysis, we used an interquartile range (IQR) upper hinge of +3.0 and a lower hinge of -3.0 and a balanced differential expression (BDE) of $\leq$ -1.5 or $\geq$ +1.5 in at least 3 out of 4 cDNA microarray experiments. Selection of transcripts from the oligonucleotide microarray experiment was based on a BDE of $\leq$ -1.4 or $\geq$ +1.4 in both the hippocampus and the cerebellum. The quantitative RT-PCR data are presented as means ± s.e.m. Statistical evaluation of the RT-PCR data was performed using the Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

To examine transcript levels in APO-SUS compared to -UNSUS rats, comprehensive gene expression profiles were determined with both oligonucleotide and cDNA microarrays. The profiles were compared between pooled RNA samples from cerebellum and hippocampus of the two rat lines at PND 9 and PND 60.

cDNA microarray analysis of APO-SUS and -UNSUS hippocampal mRNA levels at PND 60

We first investigated the hippocampus of APO-SUS and -UNSUS rats at PND 60 with Incyte NeuroGEM 2.02 cDNA microarrays. The hippocampus was chosen as primary target, because it is one of the brain regions implicated in schizophrenia-related disturbances, and is involved in emotional regulation and cognitive functions suggesting that psychotic symptoms observed in schizophrenia originate from this region (Stevens, 1973; Torrey and Peterson, 1974). Furthermore, in schizophrenic patients the hippocampus has a reduced size, shape and architecture, and displays alterations in metabolic activity and neurochemistry (reviewed by Gothelf et al., 2000; Harrison, 2004). Relative to APO-UNSUS rats, the hippocampal mossy fibers of APO-SUS rats contain increased dynorphin B protein levels (Cools et al., 1993) and the mineralocorticoid receptors (MR) in the hippocampus have significantly increased binding capacities (Sutanto et al., 1992). At the behavioural level, information processing deficits in the brain have been observed in APO-SUS rats, reflected by a reduction in prepulse inhibition of the acoustic startle response (also found to be affected in schizophrenic patients) (Braff et al., 2001; Ellenbroek et al., 1995). This process is thought to be regulated by the hippocampus (Bast and Feldon, 2003; Swerdlow et al., 2001). Finally, and most importantly - unlike other brain regions implicated in schizophrenia such as the amygdala, prefrontal cortex or accumbal region - the hippocampus is a well-defined brain region that can be dissected relatively easily from fresh brain tissue. We decided to analyse first the rats at PND 60, since at this time point the rats are considered young adults. It is not until this adult age that many of the phenotypic characteristics, such as the apomorphine susceptibility, become apparent in the rat model. The young adult time point also mimics the developmental stage at which most schizophrenic patients show their first symptoms (Jablensky and Cole, 1997; Sartorius et al., 1986). The genome-wide expression profiles of the hippocampi of male APO-SUS and -UNSUS rats at PND 60 were analysed both under basal conditions and three hours after apomorphine treatment. For the cDNA microarray analyses under basal conditions, untreated male rats were used. For the analysis following apomorphine injection, male rats of the same nests were injected with the dopamine agonist and tested for their gnawing behaviour. For three hours, the drug was allowed to exert its effect on the mRNA transcript levels, since this interval between the time of injection and transcript profile analysis has been shown by others to be sufficient to cause changes in mRNA levels (for example Adams et al., 2003; Levant et al., 1992). All APO-SUS and -UNSUS rats that were injected with apomorphine and analysed in the gnawing box met the susceptibility criteria, i.e. APO-SUS and -UNSUS rats gnawed over 500 times and less than 10 times in 45 minutes, respectively (Figure 1).

The hippocampi from ten apomorphine-treated and ten non-injected APO-SUS and APO-UNSUS rats were dissected and total RNA was isolated. The RNA samples (following either under basal or apomorphine conditions) were grouped into two pools of five isolates per genotype and treatment. These biological duplicate RNA samples were used for the generation of fluorescently labelled targets for the hybridisation to the cDNA microarrays. Of the 6,747 unique transcripts represented on the arrays, 82.7%

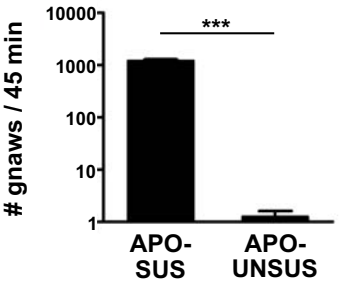


Figure 1. Effect of apomorphine injection (1.5 mg/kg) on the gnawing behaviour of the APO-SUS and -UNSUS rats used in the microarray experiments

The number of gnaws was measured for 45 minutes. ***: $P < 0.01$; n = 10 per rat line, with all rats from different nests, plus s.e.m.

were detectable in duplicate experiments (*i.e.* a hybridisation signal intensity of at least 2.5-fold above background), and most signal intensities were highly similar between the APO-SUS and -UNSUS samples, both under basal conditions and after apomorphine treatment (Figure 2). From the data, genes that showed a balanced differential expression of ≤ -1.5 or ≥ +1.5 in replicate hybridisation experiments were selected. Of these, differentially expressed genes that fell outside an inter-quartile range of 3 from the upper or lower hinge in 3 out of 4 hybridisation experiments were selected for validation experiments. Seven differentially expressed genes (~0.1% of the total number of genes on

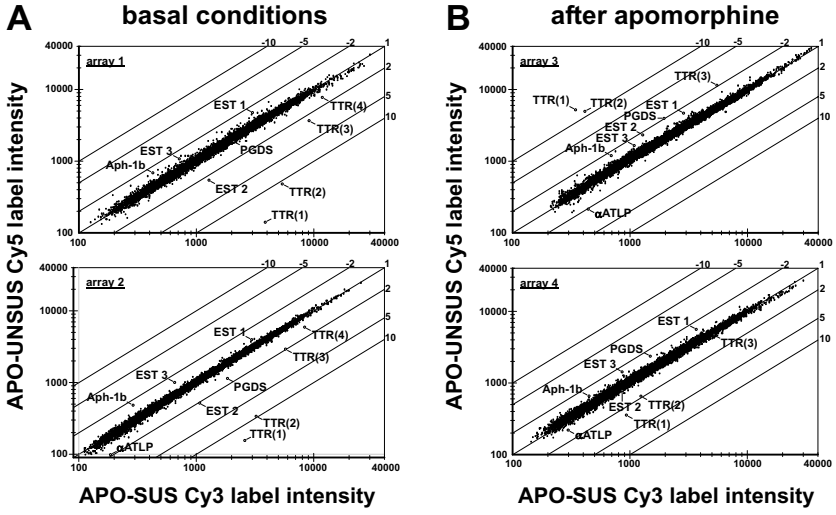


Figure 2. Scatter plots of PND 60 APO-UNSUS versus -SUS hippocampal hybridisation signal strengths on the cDNA microarrays

Log-log graph of the Cy3 (APO-SUS; x-axis) and Cy5 (APO-UNSUS; y-axis) signal values of 6,747 unique transcripts present on the microarray comparing pools of five hippocampi per microarray. The abundance of the transcripts was tested both under basal conditions (A) and three hours after apomorphine injection (B). The two graphs in (A) represent biological duplicates; same for (B). The spots with a name reference correspond to genes that were differentially expressed reproducibly (based on a 3-fold interquartile range threshold). The numbers between brackets indicate different probe sets for the same transcript.

Table 2. Differential hybridisation intensities of PND 60 hippocampal APO-SUS versus -UNSUS rat samples on the cDNA microarrays
(BD: below detection)

		fold change			
		basal conditions		after apomorphine	
Gene	Incyte ZooSeq number	array 1	array 2	array 3	array 4
TTR	700491883	28.0	16.9	-15.0	2.7
PGDS	700861868	1.1	1.6	-2.0	1.6
Aph-1b	700251056	-1.6	-1.6	-1.7	-1.4
αATLP	700289556	BD	1.9	2.1	1.4
EST1	700697881	-1.6	-1.3	-1.6	-1.5
EST2	700938990	2.4	2.1	-1.8	1.1
EST3	700253034	-1.5	-1.5	-1.5	-1.6

the array) met these criteria: *transthyretin* (*TTR*, also known as *prealbumin*), *prostaglandin D synthase* (*PGDS*, also known as β-*trace*), α-*anti trypsin-like protein* (α*ATLP*, also known as serine proteinase inhibitor *HongrES1*), the γ-secretase component *Aph-1b*, and three expressed sequence tags encoding unknown proteins (*EST1*, *EST2* and *EST3*). The differences in hybridisation intensities of each of these transcripts on the cDNA microarrays are listed in Table 2 and the corresponding sequences spotted on the microarray are listed in Appendix 1.

Oligonucleotide microarray analysis of hippocampal and cerebellar mRNA levels in PND 9 APO-SUS and -UNSUS rats

In addition to the cDNA microarray studies, we decided to examine gene expression profiles of hippocampal and cerebellar mRNA levels in PND 9 APO-SUS and -UNSUS rats with the oligonucleotide microarrays Rn_U34A from Affymetrix. The Incyte NeuroGEM cDNA microarrays used in the expression profiling at PND 60 contained genes representing only about one-third of the total number of transcripts. In addition, many potentially interesting transcripts were not present on the array (such as the *dopamine transporter*, *dopamine receptors 1* to *5*, *serotonin receptors 1a* and *2a*, *NMDA receptor subunits 1*, *2A* and *2B*, *brain derived neurotrophic factor*, and *dynorphin B*). Therefore, it was decided to perform these sets of microarray experiments with the Affymetrix oligonucleotide arrays. Although also this array contained only about one-third of the complete transcriptome, all 8,478 annotated transcripts were represented (annotation based on the Ensemble database, release Oct 2001). Also here, pooled RNA samples of five hippocampi or cerebelli from PND 9 APO-SUS and -UNSUS rats were used. On the oligonucleotide microarrays, 52.5% of all transcripts analysed in the hippocampus gave a signal above background. Similarly, the cerebellum yielded 53.9% positive hybridisation signals. However, no major differences were observed between the gene expression profiles of these tissues of the APO-SUS and -UNSUS rats

(e.g. linear regression analysis revealed r^2 values very close to 1.0, indicative of a close correlation to the mean: r^2 = 0.9917 and 0.9868, for the hippocampal and cerebellar samples, respectively; Figure 3). The transcripts with a balanced differential expression (BDE) of ≤ -1.4 or ≥ +1.4 in both the hippocampus and the cerebellum were selected for validation analysis using quantitative RT-PCR. Many of the interesting transcripts mentioned above and uniquely present on the Affymetrix oligonucleotide microarrays either did not reveal any differences between the rat lines (e.g. for *dopamine receptors 1* and *3*, and *NMDA receptor 1*), or the expression levels were too low to obtain signals above background levels (e.g. for *dopamine receptors 2* and *4*, *serotonin receptor 1A* and *NMDA receptor 2A*). The seven differentially expressed transcripts that did meet the selection criteria were: *microtubule-associated protein 2* (*MAP2*), *cyclin G*, *S-100 related protein*, *catechol-O-methyltransferase* (*COMT*), immediate early gene (IEG) *Homer 2* (or *Vesl-2*), *histone H4* and *phosphatidylinositol 3-kinase regulatory subunit p85* (*p85 PI3K*; Table 3).

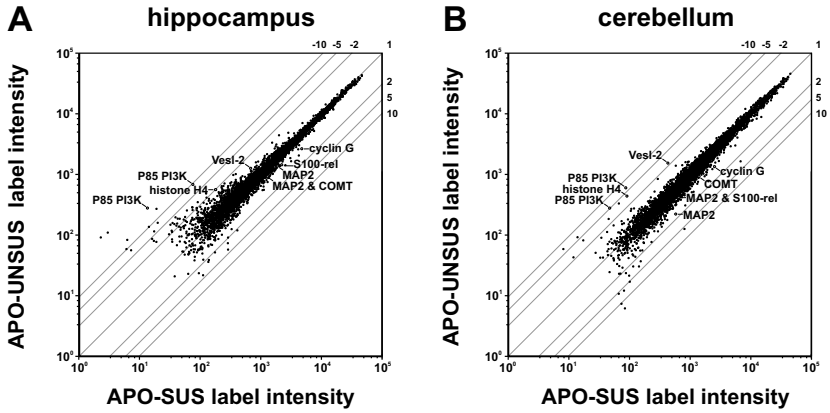


Figure 3. Scatter plots of PND 9 APO-SUS versus -UNSUS hippocampal and cerebellar hybridisation signal strengths on the oligonucleotide microarrays

Log-log graph of the APO-SUS and -UNSUS signal intensities above background levels of biotin-labelled cRNA samples of either pooled hippocampi (A) or cerebelli (B). Spots with a name reference represent transcripts used in validation and follow-up experiments.

Validation of cDNA microarray data

The validation experiments for the seven transcripts selected via the cDNA microarray experiments were performed by means of real-time quantitative RT-PCR analysis on the same RNA samples as used in the microarray experiments. The differences in *TTR*, *PGDS* and *Aph-1b* mRNA levels between the APO-SUS and -UNSUS hippocampal samples could be confirmed by the quantitative RT-PCR analysis (Figure 4). In contrast, the validation results of the *EST1*, *EST2* and *EST3* mRNA levels showed no differences between the two rat lines, while the levels of α*ATLP* mRNAs were below detection, consistent with the low hybridisation signals of α*ATLP* on the cDNA microarrays (Figure 2).

Table 3. Differential hybridisation intensities of PND 9 hippocampal and cerebellar APO-SUS (AS) and -UNSUS (AU) rat samples on the oligonucleotide microarrays

Included are the gene names, probe set codes, the normalised hybridisation signals, the present (P) or absent (A) calls and the fold difference in log(2) ratio as calculated by the Microarray Suite 5.0 software.

		hippocampus					cerebellum				
Gene	Systematic Name	AS		AU		log(2) ratio	AS		AU		log(2) ratio
MAP2	X17682_s_at	1241.5	P	976	P	-0.5	577.3	P	223.3	P	-0.8
MAP2	X53455cds_s_at	1884.6	P	1410.7	P	-0.4	634.7	P	415.9	P	-0.6
cyclin G	X70871_at	4743.3	P	2644	P	-0.7	2486.1	P	1364	P	-0.6
S-100 related	J03627_at	2197.2	P	1451.1	P	-0.6	662.2	P	424.7	P	-0.6
COMT	M93257_s_at	1301.7	P	920.4	P	-0.3	1358.6	P	917.1	P	-0.7
Vesl-2	AB007690_s_at	686.8	P	1139.7	P	0.7	428.9	P	1548.9	P	1.4
histone H4	rc_AA946439_at	182.5	A	564.7	P	1.7	91.1	A	441.8	P	1.7
p85 PI3K	D64045_s_at	13.4	A	281.7	P	2.9	46.7	A	278.7	P	2
p85 PI3K	U50412_at	75.8	A	694.5	P	2.5	86.9	A	606.3	P	1.7

The largest differences in mRNA levels between the APO-SUS and -UNSUS rats were observed for *TTR* and *PGDS*. More detailed analysis of the transcript levels of these genes in individual hippocampal samples revealed remarkably large fluctuations (Figure 5A). While in most of the APO-SUS and -UNSUS samples relatively low *TTR* and *PGDS* mRNA levels were observed, drastically increased *TTR* and *PGDS* transcripts levels were detected in only a limited number of hippocampal RNA isolates. Subsequent *in situ* hybridisation analysis of *TTR* mRNA distribution in the rat brain revealed this transcript to be exclusively and abundantly expressed in the choroid plexus, an epithelial cell layer present in the ventricles and surrounding the hippocampus (Figure 5B). Quantitative RT-PCR analysis of isolated choroid plexus tissue showed no significant differences in the *TTR* mRNA levels of APO-SUS and -UNSUS rats (Figure 5C). From these results, we conclude that the large differences in *TTR* (and presumably also in *PGDS*) mRNA levels between the pools of APO-SUS and of APO-UNSUS hippocampi resulted from their expression in contaminating choroid plexus and thus from a dissection artefact during the isolation of the hippocampus. Thus, the only reproducible result from the cDNA microarray experiments was the observed reduction in *Aph-1b* mRNA levels in APO-SUS compared to -UNSUS rat tissue samples (see below).

Validation of oligonucleotide microarray data

Similar to the cDNA microarray validations, the transcripts selected from the oligonucleotide microarrays were subjected to validation using quantitative RT-PCR. Now, however, individual RNA samples were used instead of pooled RNA. These experiments could not confirm any of the differences indicated from the oligonucleotide microarray data (not shown). Thus, the oligonucleotide microarray experiments did not reveal any new differentially expressed genes.

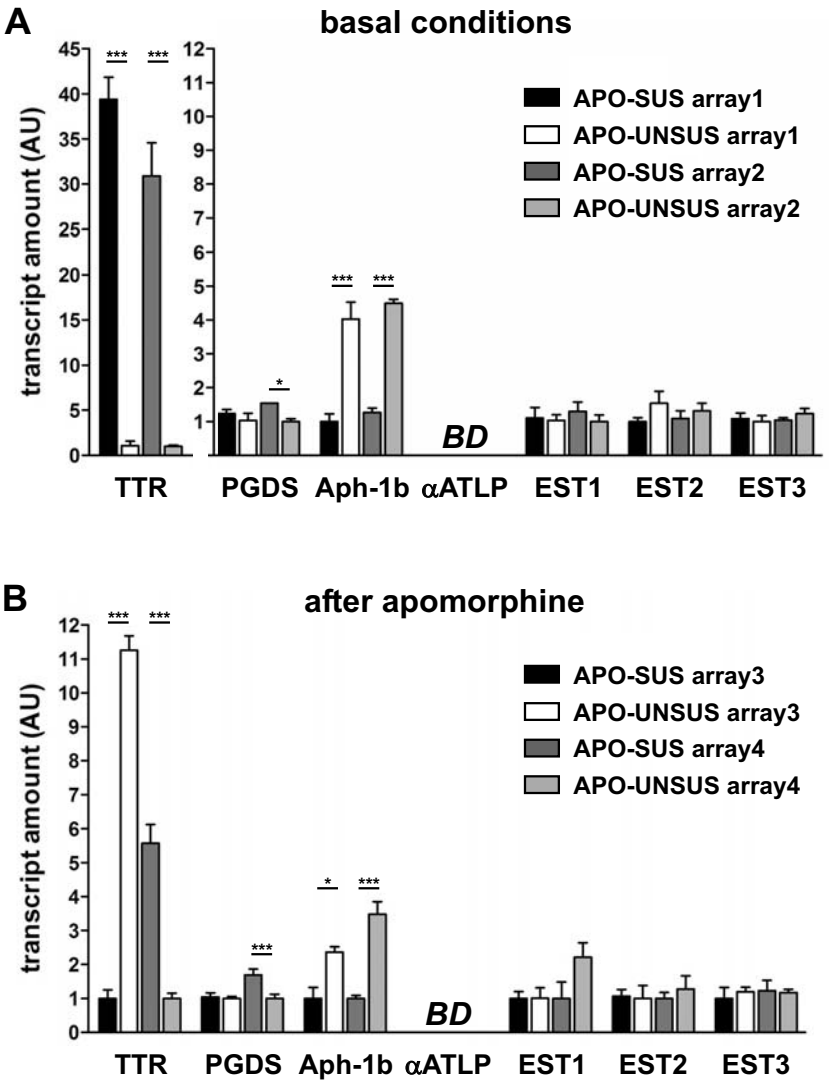


Figure 4. Real-time quantitative RT-PCR validation of cDNA microarray data

The seven genes that were found on the cDNA microarrays to be differentially transcribed were tested for further validation via quantitative RT-PCR. The same RNA pools were used as in the expression profiling analysis, under basal conditions (A) and after apomorphine administration (B). Results were normalised towards β-*actin* and are expressed as arbitrary units (AU). The differences in transcript levels for *TTR*, *PGDS* and *Aph-1b* could be confirmed via this technique, while the levels of *αATLP* mRNA were below detection (BD), and the *EST1*, *EST2* and *EST3* mRNA levels did not appear to differ between the rat lines. Experiments were preformed in triplicate and shown as mean plus s.e.m.; *: $P < 0.05$; ***: $P < 0.001$.

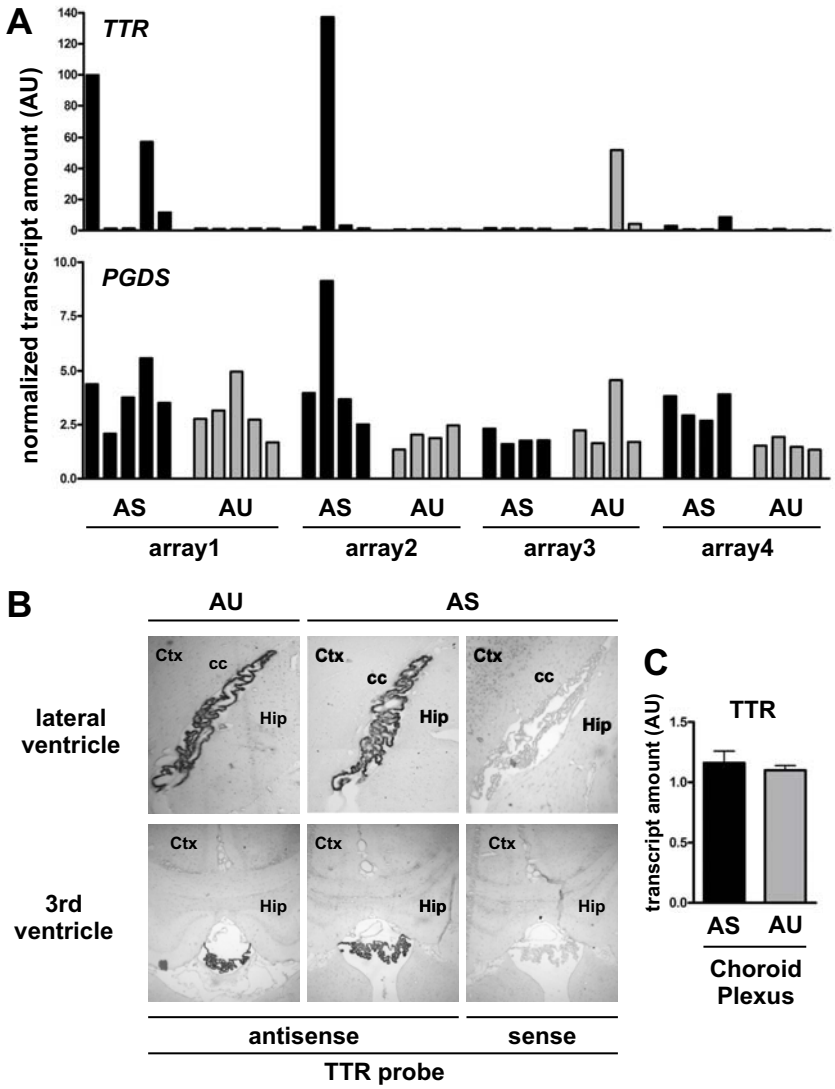


Figure 5. *TTR* and *PGDS* mRNA levels in the APO-SUS / -UNSUS rats

(A) Real-time quantitative RT-PCR analyses of *TTR* and *PGDS* mRNA levels in individual hippocampal samples from APO-SUS (AS) and -UNSUS (AU) rats. Results were normalised towards β-*actin* and are expressed as arbitrary units (AU). Note the large differences in *TTR* mRNA levels (and to a lesser extent also for *PGDS* mRNA levels) between some of the individual samples. (B) *In situ* hybridisation results for *TTR* in Wistar rats. Hybridisation of the *TTR* DIG-labelled antisense RNA probe was observed only in the choroid plexus, an epithelial cell layer surrounding the hippocampus (Hip; left panel). No signal for *TTR* was found within the hippocampus. Hybridisation of a *TTR* DIG-labelled sense control RNA probe revealed no background hybridisation (right panel); Ctx: cortex; cc: corpus callosum. (C) Real-time quantitative RT-PCR on the choroid plexus *TTR* mRNA levels in APO-SUS and -UNSUS rats. No significant differences for *TTR* mRNA could be observed; β-actin was used for normalisation.

Aph-1b mRNA levels in the APO-SUS and APO-UNSUS rats

To further analyse the *Aph-1b* mRNA levels in the rat model, individual hippocampal RNA samples were analysed by quantitative RT-PCR. Relative to APO-UNSUS, in all APO-SUS samples the *Aph-1b* transcript levels were reduced (2.2- and 3.1-fold average reduction in basal and apomorphine-treated APO-SUS rats, respectively; $P < 0.05$) (Figure 6A). Analysis of the distribution of the *Aph-1b* transcript in the hippocampus using *in situ* hybridisation revealed strong signals in the dentate gyrus as well as in the pyramidal cell layers of the CA1-CA3 regions of the hippocampus, while the choroid plexus gave a much lower signal. This indicated that the differences observed in *Aph-1b* mRNA levels between APO-SUS and -UNSUS hippocampal isolates - in both quantitative RT-PCR and microarray experiments - were not caused by contaminating choroid plexus material (Figure 6B). Furthermore, semi-quantitative RT-PCR analysis of *Aph-1b* mRNA in various tissues (olfactory bulb, hippocampus, cortex, cerebellum, [hypo]thalamus, pituitary, heart, lung, liver, spleen, kidney, adrenal gland, small intestine, thymus, eye, tongue, muscle, stomach, testis and epydidimus) revealed expression of the transcript in all tissues analysed (data not shown). Therefore, the reduction of *Aph-1b* mRNA levels in the hippocampus of APO-SUS relative to -UNSUS rats appeared to be the only reproducible difference found in the gene expression profiles of the two rat lines.

Further analysis of transcript levels of the Aph-1 family members in APO-SUS and -UNSUS rats

From extensive BLAST searches against the rat EST and genome database (via http://www.ncbi.nlm.nih.gov/*B*LAST), the complete coding sequence of the rat *Aph-1b* gene was derived. The gene consists of 6 exons and is mapped to rat chromosome 8q24 (LOC503216). A tBLASTn search with the Aph-1b amino acid sequence revealed a highly similar (56.2% amino acid identity) paralogue sequence, later termed Aph-1a (Figure 7A). Similarities were also found in the build-up of the genes, with highly similar exon sizes between the two genes (Figure 7B). Quantitative RT-PCR revealed that in the hippocampus of PND 9 APO-SUS and -UNSUS rats the *Aph-1a* mRNA levels were not different, while the *Aph-1b* mRNA levels were significantly reduced in the APO-SUS rats (Figure 7C). Thus, the *Aph-1a* mRNA levels did not compensate for the reduced levels of its paralogue. Furthermore, the differences in *Aph-1b* mRNA were observed in both male and female rats, indicating that there are no gender differences for the *Aph-1b* mRNA levels. In addition, this experiment showed that the differences in hippocampal *Aph-1b* mRNA levels can be observed both at PND 9 and PND 60.

All data presented thus far was generated from the replicated APO-SUS and -UNSUS rat lines. These 'newer' rat lines are independent phenotypic replications that have been generated ten years after developing the original APO-SUS and -UNSUS lines (Cools et al., 1990; Ellenbroek et al., 2000). To test whether the original rat lines displayed similar differences in *Aph-1b* mRNA levels, quantitative RT-PCR was performed on PND 9 hippocampal RNA samples from these lines. The data showed that the reduction in *Aph-1b* mRNA levels in the APO-SUS relative to the -UNSUS rats was not restricted to the replicated rat lines, but was also observed in the original rat lines (Figure 8).

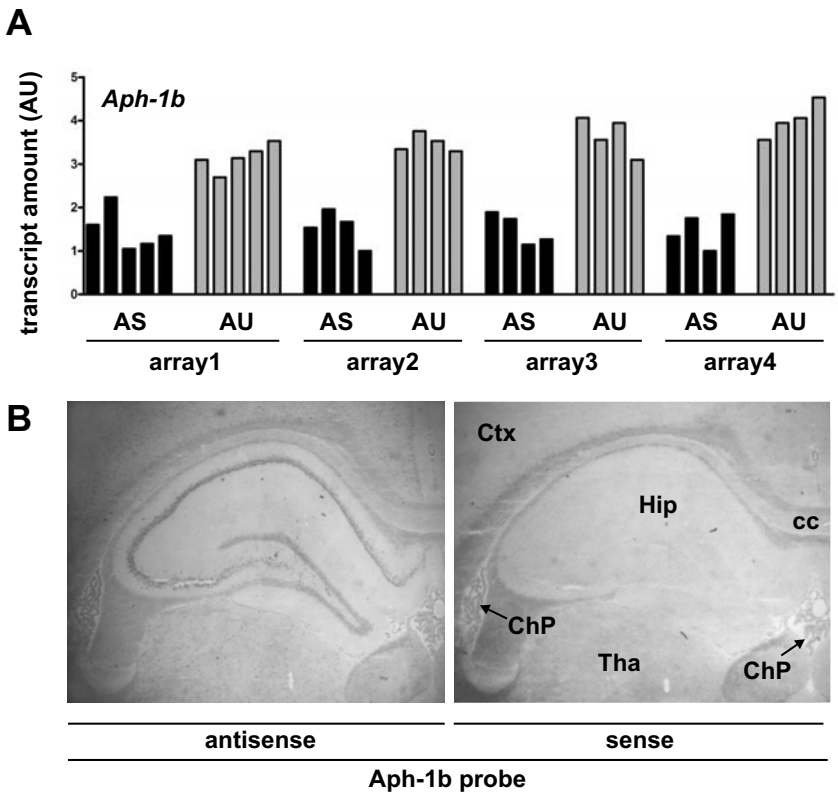
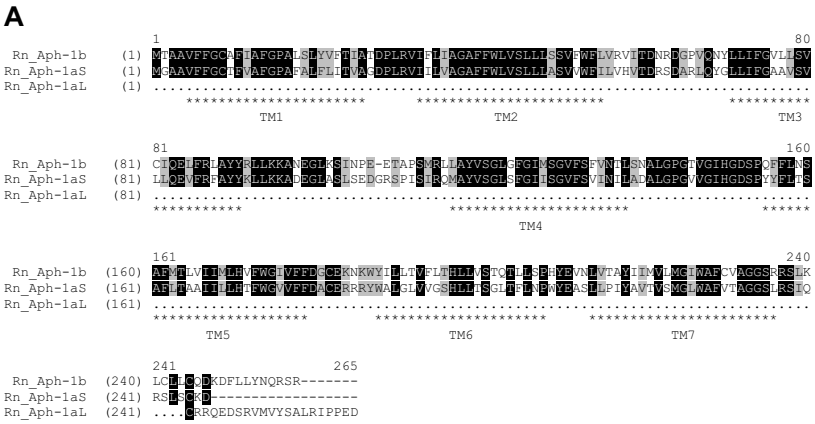


Figure 6. *Aph-1b* mRNA levels in the APO-SUS / -UNSUS rats

(A) The *Aph-1b* mRNA levels in individual PND 60 APO-SUS and -UNSUS rat hippocampal samples were determined using real-time quantitative RT-PCR. The abundance of the *Aph-1b* transcripts normalised towards β-*actin* mRNA levels was examined both under basal conditions (left panel) and three hours after apomorphine injection (right panel). Results are expressed as arbitrary units (AU). Student *t*-test revealed a significant 2.2- and 3.1-fold reduction in basal and apomorphine-treated APO-SUS rats, respectively; $P < 0.05$. (B) *In situ* hybridisation results for the *Aph-1b* mRNA distribution in the rat hippocampus (Hip) and choroid plexus (ChP). The *Aph-1b* DIG-labelled antisense RNA probe was predominantly detected in the dentate gyrus and in the pyramidal cell layers of the CA1-CA3 regions of the hippocampus, with much lower signals in the choroid plexus. Other brain regions with strong hybridisation signals were the olfactory bulb and cerebellum, while moderate signals were observed throughout the rest of the brain (data not shown). Hybridisation of a control *Aph-1b* DIG-labelled sense RNA probe revealed no background signals (right panel); Ctx: cortex; cc: corpus callosum; Tha: thalamus.

Analysis of the genetic locus of human, mouse and rat Aph-1b

A comparison of the rat *Aph-1b* mRNA sequence with the human genome database sequences revealed the human locus for the *Aph-1b* gene to be 15q22.2. The human gene consists also of six exons and is surrounded by the *Ras-associated small GTPase Rab8b* (15q22.2) gene at the centromeric side and *carbonic anhydrase XII* (*CA12*) (15q22.2) and the *HECT domain and RCC1 domain 1* (*HERC1*) gene (15q22.31) at the telomeric side of



B

	exon 1	*intron*	exon 2	*intron*	exon 3	*intron*	exon 4	*intron*	exon 5	*intron*	exon 6	*intron*	exon 7
Aph-1b	**113**	*1224*	**171**	*6087*	**71**	*774*	**123**	*6190*	**128**	*2796*	**168**	-	-
Aph-1aS	**113**	*542*	**171**	*149*	**74**	*228*	**123**	*153*	**128**	*400*	**135**	-	-
Aph-1aL	**113**	*542*	**171**	*149*	**74**	*228*	**123**	*153*	**128**	*400*	**124**	*331*	**65**

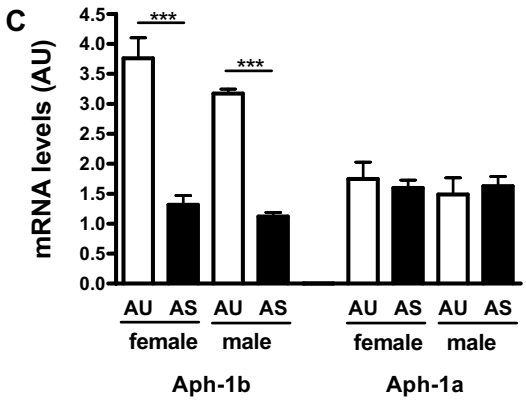


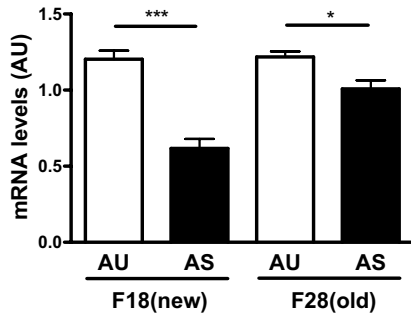
Figure 7. The Aph-1 family

(A) Alignment of the amino acid sequences of the rat Aph-1a and -1b proteins. The *Aph-1a* gene can be alternatively spliced into two proteins with distinct carboxy-termini, Aph-1aS and -1aL. Predicted transmembrane regions are indicated by an asterix (black background: idential amino acids, grey background: conserved amino acids, dots: sequence identical to Aph1aS). (B) Comparison of the exon and intron sizes of the rat *Aph-1a* and *-1b* genes. The base pair counts of the individual exons and introns are shown; the base pair count of the first exon does not include the 5' untranslated region (UTR), while for the count of last exon the 3'-UTR is excluded. (C) Real-time quantitative RT-PCR analysis of *Aph-1b* and *-1a* mRNA levels in hippocampal samples of PND 9 male and female APO-SUS and -UNSUS rats. The primer sets used specifically detected all *Aph-1b* or *-1a*-related transcripts. Whereas both male and female samples show significant reduction in APO-SUS relative to -UNSUS rats, no significant differences were observed in *Aph-1a* mRNA levels. Results (n = 3, plus s.e.m.) were normalised towards β-*actin* and are expressed as arbitrary units (AU); ***: $P < 0.001$.

the *Aph-1b* locus. A similar chromosomal distribution of the genes was found on mouse chromosome 9d and rat chromosome 8q24. To analyse whether in the genomic locus the transcript level of a gene other than *Aph-1b* was affected in the APO-SUS/-UNSUS

Figure 8. Comparison of *Aph-1b* mRNA levels in male hippocampus of the original and replicated PND 9 APO-SUS and -UNSUS rat lines

Real-time quantitative RT-PCR analysis of *Aph-1b* mRNA revealed significantly reduced levels in both the original and the replicated rat line samples. Results (n = 6, plus s.e.m.) were normalised towards β-*actin* and are expressed as arbitrary units (AU); *: $P < 0.05$; ***: $P < 0.001$.



rat model, the mRNA levels of the *Rab8b*, *CA12* and *HERC1* genes were determined in the APO-SUS and -UNSUS hippocampal samples. Quantitative RT-PCR analysis of the expression levels of these genes in the rat model did not reveal any differences (Figure 9), indicating that *Aph-1b* is the only affected gene in this locus.

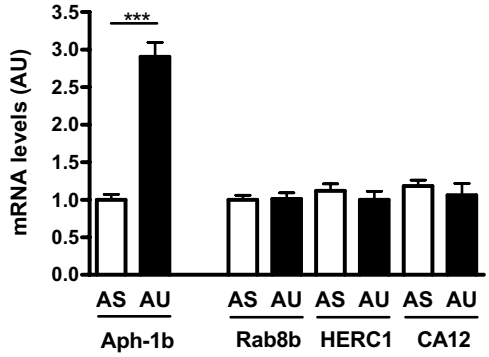


Figure 9. Analysis of the transcript levels of genes near the *Aph-1b* locus in the hippocampus of PND 9 APO-SUS and -UNSUS rats by real-time quantitative RT-PCR

The primer sets used detected the neighbouring *Rab8b*, *CA12* or *HERC1* transcripts. Beside the reduced *Aph-1b* mRNA levels, no significant differences were observed. Results (n = 6, plus s.e.m.) were normalised towards β-*actin* and are expressed as arbitrary units (AU); ***: $P < 0.001$.

Discussion

The microarray technology is predicted to have a great impact on finding causes or proper treatments for a variety of diseases (e.g. Archacki and Wang, 2004; Meloni et al., 2004; Raetz and Moos, 2004). However, for complex neurodevelopmental disorders, such as schizophrenia, the variability in sample and patient conditions severely impedes

any transcript analysis. In the present study, we analysed the mRNA expression profiles of a well-established rat model for schizophrenia-related disturbances, the APO-SUS/-UNSUS rat model. Our initial cDNA microarray comparison of hippocampal RNA samples from APO-SUS and -UNSUS rats at PND 60 revealed only a limited number of differences, both under basal conditions and after apomorphine administration. Further analysis of PND 9 hippocampal and cerebellar mRNA profiles using oligonucleotide microarrays showed no major alterations in transcript levels in the rat model. The fact that only a limited number of differences were found could be caused by the cellular complexity of the tissues analysed. Both the hippocampus and cerebellum are known to consist of many different cell types and the analysis of the whole tissues will thus dilute any differences that may be present only in a subset of cells or cell types (Mackler et al., 1992; Zawar et al., 1999). The most striking difference we observed in the hippocampal cDNA microarray analyses was the differential mRNA levels of *TTR*. Detailed validation experiments learned that the *TTR* transcript is present at high levels, but exclusively in the choroid plexus, an epithelial cell layer surrounding all ventricles of the brain. The hippocampus is in direct contact with both the 3rd and the lateral ventricles, and in our experiments some of the hippocampal isolates must have been contaminated by partly co-dissected choroid plexus material. High *TTR* mRNA levels have been reported to be present in the hippocampus (Long et al., 2003; Puskas et al., 2003; Rho et al., 2005). However, based on our results, most likely these high levels have been caused also by dissection artefacts. The observed large differences in *PGDS* mRNA levels among individual samples are probably the result of the same artefact. Like TTR, PGDS is one of the major protein constituent of cerebrospinal fluid and in the central nervous system it is mainly synthesised in the arachnoid cells and the choroid plexus, but unlike TTR also to some extent in oligodendrocytes (Kawashima et al., 2001). This may explain why the observed fluctuations in *PGDS* mRNA levels in the individual hippocampal isolates were somewhat smaller than for *TTR* mRNA. A more accurate dissection may be accomplished by the use of laser dissection microscopy on brain sections to isolate more specific brain regions. This technique allows the selection of certain tissue regions and even the isolation of only a small subset of cells for expression profiling studies, thereby drastically increasing the chances of finding differences. The major disadvantage in using such a small number of cells for microarray expression analysis is that the yield of RNA is too low for direct microarray experiments and thus amplification of the RNA is necessary. Although after our experiments had been conducted, techniques have been greatly improved, per microarray hybridisation several micrograms of total RNA are still needed. Since it is extremely difficult to amplify all transcripts with the same efficiency, such an additional step is prone to introduce undesirable differences in the expression profiles.

Detailed comparisons of results obtained through either cDNA or oligonucleotide microarray experiments have shown that data generated from oligonucleotide microarrays are more reliable for interrogating changes in gene expression than data from long cDNA microarrays (Li et al., 2002; Woo et al., 2004). In our experiments, for both types of microarrays we initially observed similar percentages of altered gene transcripts (for both microarrays ~0.1%). However, follow-up validation experiments

argued against all but one difference, namely a 2.6-fold reduction of *Aph-1b* mRNA levels in APO-SUS relative to -UNSUS rats, a difference discovered using the cDNA microarray. It should be noted that the *Aph-1b* transcript was not present on the oligonucleotide microarrays and therefore not detected in this profiling study at PND 9. However, this does not imply that the affected *Aph-1b* transcript levels represent the only transcriptional difference between the two rat lines. First, roughly only one third of all rat gene transcripts were present on the microarrays used, so differences could be hidden in the other two thirds of the transcripts. Secondly, expression microarrays have their limitations in detection sensitivity. Differences have to be of a reasonable order (at least ~40% increase or decrease) to allow detection above the technical background spreading in signal intensities. Nevertheless, only small differences can have a great physiological impact, especially in *in vivo* situations. Moreover, for microarray analysis the amount of a transcript has to be higher than a certain threshold level to obtain hybridisation signals above background. Differences in low-abundant transcripts, encoding for example pivotal enzymes or receptors of important pathways, may therefore be undetectable.

Detailed PCR-based transcript analysis of the *Aph-1b* gene confirmed the reduced levels in APO-SUS compared to -UNSUS rats. Differences were found in the hippocampus at PND 9 and 60, and in both male and female rats, suggesting a general or basic cause. Furthermore, since the *Aph-1b* mRNA levels were reduced in both the original and replicated APO-SUS lines the reduced expression levels are likely to be responsible for the phenotypic characteristics of the APO-SUS rats. The APO-SUS and -UNSUS rat lines have been generated independently of each other and with a ten-year interval (Ellenbroek et al., 2000).

The reduced expression of *Aph-1b* in the APO-SUS relative to the -UNSUS rats could have various origins: the expression of a transcription factor regulating the *Aph-1b* gene may be different, the locus of the *Aph-1b* gene could be affected (for example, by a single nucleotide polymorphism [SNP], deletion or mutation), the epigenetic status of the locus could vary, the *Aph-1b* mRNA could have a different stability, or the pathway in which the Aph-1b protein is involved could be affected (with a feedback on *Aph-1b*). Our analysis of the transcript levels of neighbouring genes of the *Aph-1b* locus (on rat chromosome 8q24) did not reveal any differences between APO-SUS and -UNSUS rats, indicating that the affected transcript levels in this locus are confined to the *Aph-1b* gene.

Recently, it has been found that Aph-1b is part of the γ-secretase complex, an enzyme responsible for the intramembrane cleavage of many type I transmembrane proteins (De Strooper, 2003; Francis et al., 2002; Goutte et al., 2002). The complex, consisting of presenilin, nicastrin, PEN-2 and Aph-1, is notoriously known for its pathological involvement in Alzheimer's disease (Price et al., 1995; Sherrington et al., 1995). In future experiments, we will examine whether in the APO-SUS/-UNSUS rats the differences observed in *Aph-1b* mRNA levels are accompanied by alterations in Aph-1b protein levels. Furthermore, it will be highly interesting to investigate whether differences can be found for the other components of the γ-secretase complex as well, or even for the enzymatic functionality of the γ-secretase complex. Thus far, we have analysed the mRNA levels for

the paralogue of *Aph-1b*, termed *Aph-1a*, and found no compensation for the reduction of *Aph-1b* levels in the APO-SUS rats. Future research will thus provide more insight into all components and the functioning of γ-secretase in our rat model, and whether Aph-1b or γ-secretase is truly causative of the schizophrenia-related phenotype.

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Appendix 1: List of sequences spotted on cDNA microarray

Incyte ZooSeq number 700491883 (TTR):

GCCTGTTCCTCCTCTGCCTCGCTGGACTGATATTTGCGTCTGAAGCTGNCCCTNTAGG
GTGCTGANAAGAATCCAAGTNTCNTCTGATGGTCAAAGTCNTGGANNCNNTCCNAAG
NAGNCCTGNNGTT

Incyte ZooSeq number 700861868 (PGDS):

GTGGAGGCCAAGCCTGGTTCATAAATAGGGTCTCCACGGTGGCCTCTGCTCCATCTGC
CCACAGTCTTCCTTGCTTTGCCCACGTTGCTGGCCTCAGGCTCAGACACCTGCTCTAC
TCCAAGCAAATGGCTGCTCTTCCAATGCTGTGGACCGGGCTGGTCCTCTTGGGTCTCT
TGGGATTTCCACAGACCCCAGCCCAGGGCCATGACACAGTGCAGCCCAACTTTCAACAA
GACAAGTTCCTGGGGCGCTGGTACA

Incyte ZooSeq number 700251056 (Aph-1b):

AAAAAAGCCAGCGAGGGTTTGAAAAGCATAAACCCTGAGGAGACAGCACCTTCGATGC
GACTGTTGGACTATGTTTCTGGCTTGGGCTTTGGAATCATGAGTGGACTGTTTTCCTT
TGTGAACACCCTGTCTAATGCCTTGGGGCTAGTCACAGTGGGCATTCACGGTGATTACC
CTCAGTTCTTCATTAATTCACCTTTCATGACGCTGGTTATCATAATGCTGCACGTGTTC
TGGGGAATCGAGTTTATTAACGGCTGTGAGAAGAATAAGTGG

Incyte ZooSeq number 700289556 (α-ATLP / SPI-1 serine protease inhibitor (AA 1-403)):

GCGGGATCTACACCGGAAGTTTGCTCTTTATAGACAAGACTCTAAAGCCAGCAAAGACT
TTTGTCAAACTAGCCAACAGCTCCTACAACAGCAATGTCGTCCTCATCTCTTTTGGGAA
CTACGGGTTGGCCCAAAAGCAGATAGACTTGGCCATTCGTGCCAGGACCCACGGCAAA
ATTACAAAGCTGCTGAGAATTCTGAAGCCACCAACTAACTTGTTTCTGGCCAATTATAAT
TTCTTTAAAGGGAAGTGGAAATACCCCTTTAACCGGAAGCACACAAGGATGAGGTACTT
CTGGTTAGAAGATG

Incyte ZooSeq number 700697881 (EST1 / MOUSE, CLONE MGC:6948):

GGCGCACGCCGGTAGGATCTGCCTCCCAAATGTTGGGATTAAAAGTGTGAGCTGCCAC
TGCACAGCATCGGAATTCTTTCTATTGCTTCTTCAAAGATTCATTCCCATGGCCACTAT
GATGATGACACGTGATGGGGAGATGGGAAAGACAGAAAACAGTGGTGTATGCAGACATG
GTGGTGGTGACAGGTGAGAGAGGGTTGACCTGCTGTCATTGCTCAGTAAGGTCCACAG
CATGGTGTGTGGC

Incyte ZooSeq number 700938990 (EST2 / CGI-84 protein):

CAAACAATTACATTTTAAAATAAAATTGAGAGTTACCCAAACAGTTACCCAATCTGTCTT
ACAAAGCTTATAAAAGTGAATTGGATATAATTAGCCAAATGAAGAAAGAAGACACTGAAT
TAATAACACTTTACAGTGATGAAAATATACTTGGTCTTTCTTCCAAGAACTGAGATTTTC
TCAGTATGAATTATAAGTCTGTGCTGGAATTTTGGATTGCATTAATATACACATTAAACT
CTGTAAATGAAGCCATTCTT

Incyte ZooSeq number 700253034 (EST3 / Incyte EST):

CAGTAGGTTAGAATACCCAGTGTGGCCTGATAGGGTAGGAGAAAGTTCCTAGATGTGAA
GGTTGACGTTCATTCTTGGAAGTTTGGTGGGTGGTTGGAGGGGTGTTGGGGATCAGG
AAGGGGGCTAAAACTCAATGTTTATTCACCTACCCATGCCAAAAAACTAGTATTTTGTAA
TGGTTTTGTAAGAGGAGTAGACAGACGAATTAAGTTTTAGCCGGTTCCCCCCTAGTCCC
ATCC

Chapter 3

GENE-DOSAGE EFFECT ON GAMMA-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 TH2 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 (Degen et al., 2004; Ellenbroek et al., 2000). 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 (PND 60-70) 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. 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. β-*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'-GTGATTCTCCTCAGTTCTTCCT TAATTC and 5'-GCCCATGAGCACCATGATTATAT; *Aph-1a* related (547-670): 5'-

AGAGGAGACGGTACTGGGCTTT and 5'-ATGGAAACGGTGACTGCATAGA; *presenilin-1* (259-378): 5'-GTTCCTGTGACCCTCTGCATG and 5'-GCCTACAGT-CTCGGTGTCTTCTG; *presenilin-2* (1093-1218): 5'- GGAGACTTCATCTTCTA CAGCGTTCT and 5'-GAGCAGCAGGAGGGTGAGAC; *nicastrin* (504-622): 5'-TGGCTTGGCTTATGACGACTT and 5'-TCGGTGCAGAGCCATTCTG; *Pen-2* (14-162): 5'-GGGTGTCCAATGAGGAGAAGTT and 5'-TTGATTTGGCTCT-GCTCTGTGTA; β-*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 *-1b'*, 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 (Cools and Ellenbroek, 2002; Ellenbroek and Cools, 2002; Ellenbroek et al., 2000). 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

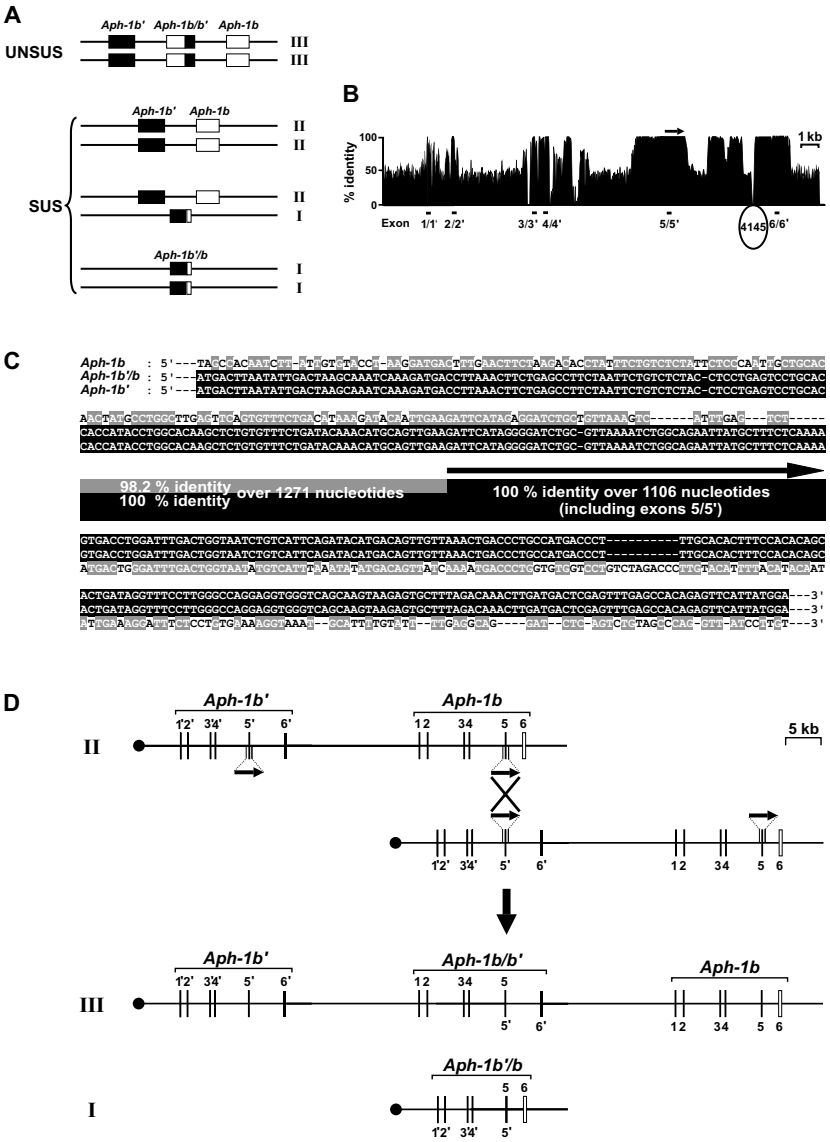
We next considered the possibility that the different *Aph-1b* mRNA expression levels in APO-SUS and -UNSUS were the result of a genomic DNA mutation. Database searches revealed that the rat (on chromosome 8q24) and mouse (on chromosome 9c) contain in tandem two *Aph-1b*-related copies (designated here *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

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.

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 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

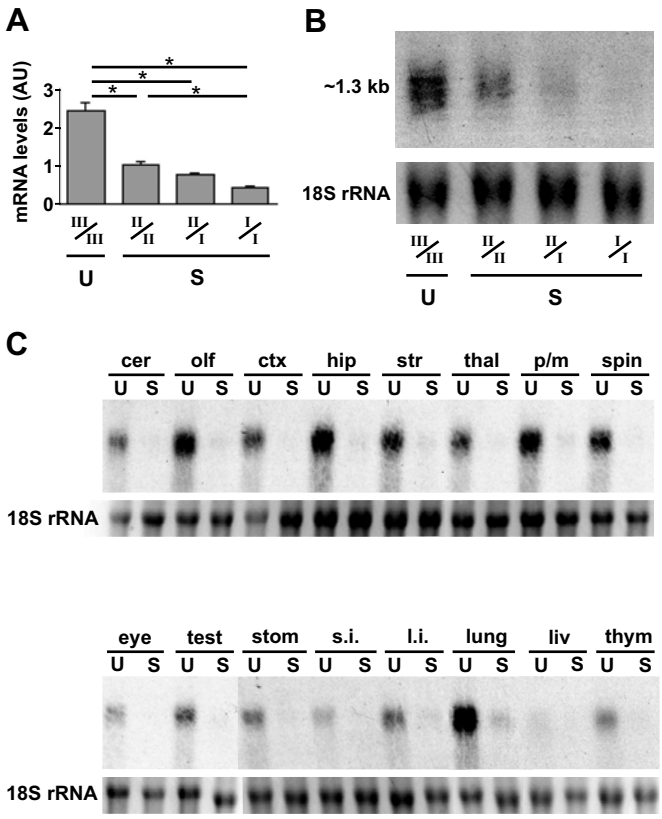


Figure 2. *Aph-1b* mRNA expression in the hippocampus and other tissues of PND 9 APO-UNSUS (U) and APO-SUS (S) rats

(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.

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

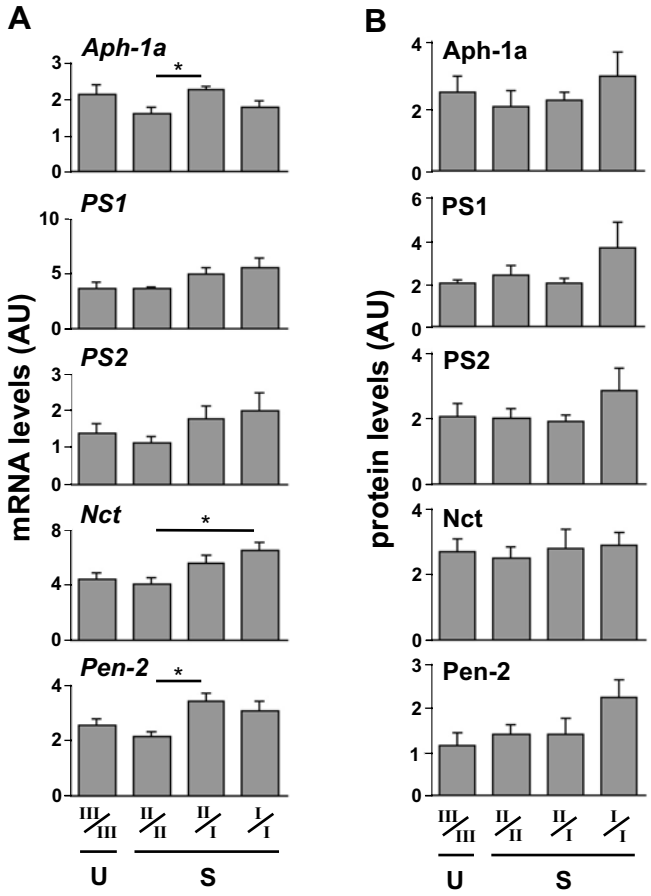


Figure 3. mRNA and protein expression of gamma-secretase components in the hippocampus of PND 9 APO-UNSUS (U) and -SUS (S) rats
(A) Quantitative RT-PCR analysis of *Aph-1a*, *presenilin-1* (*PS1*) and *-2* (*PS2*), *nicastrin* (*Nct*) and *Pen-2* mRNAs in the hippocampus of PND 9 APO-UNSUS and APO-SUS (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).

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.

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 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). 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.

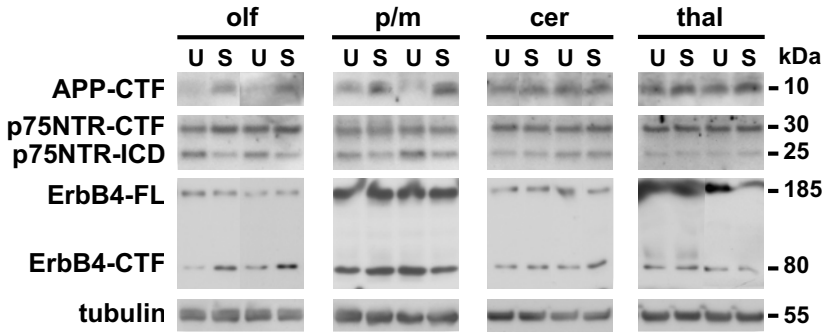


Figure 4. Western blot analysis of the cleavage products of gamma-secretase substrates in PND 2 APO-UNSUS (U, III/III) and APO-SUS (S, I/I) rat tissues

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 (-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).

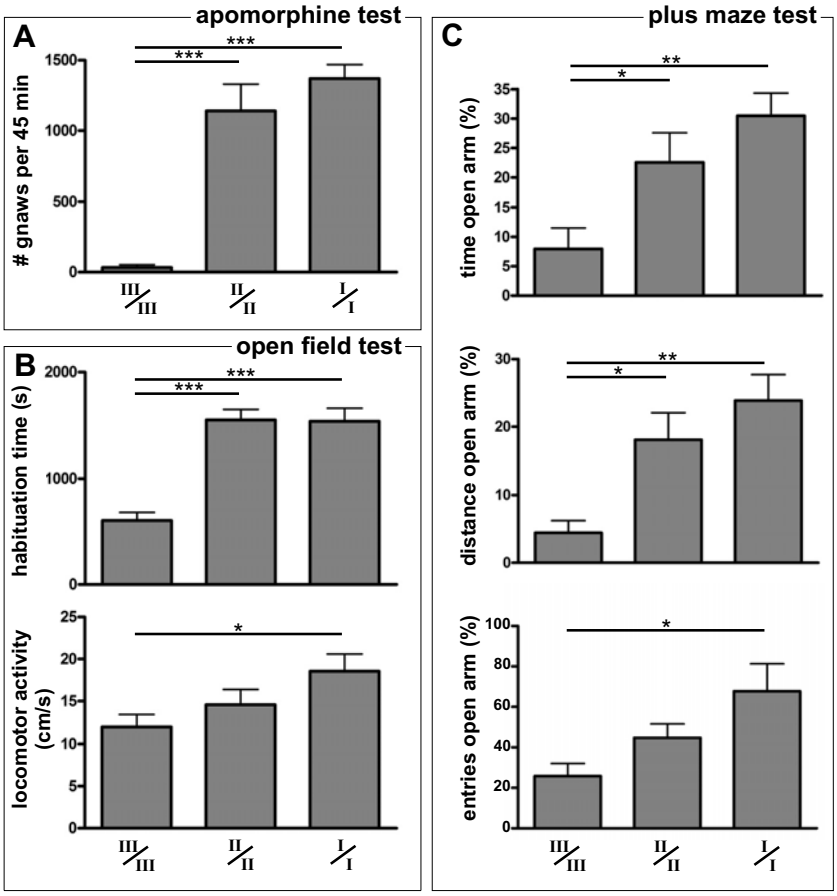


Figure 5. Behavioural phenotypic analysis of adult rats with I/I, II/II and III/III genotypes

(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 (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$).

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*.

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 (De Strooper, 2003; Fortini, 2002). 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.

Acknowledgements

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Chapter 4

ONTOGENIC REDUCTION OF APH-1B mRNA AND GAMMA-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., 2005). 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. 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., 2005). 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., 2005), 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 (ED 0). 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., 2005). The following primers were used: *Aph-1b* (448-671): 5'-GTGATTCTCCTCAGTTCTTC CTTAATTC and 5'-GCCCATGAGCACCATGATTATAT; *Aph-1aL* (572-779):5'-CCTGGTAGTTGGGAGTCACCTT and 5'-CGCAGGGCAGAGTACACCAT; *Aph-1aS* (572-761): 5'-CCTGGTAGTTGGGAGTCACCTT and 5'-CGGTGCAG-

TCCAGGTAGTCAGT; β-*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., 2005). 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 normalised 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 analysed 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 analysed 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 and D). 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.

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

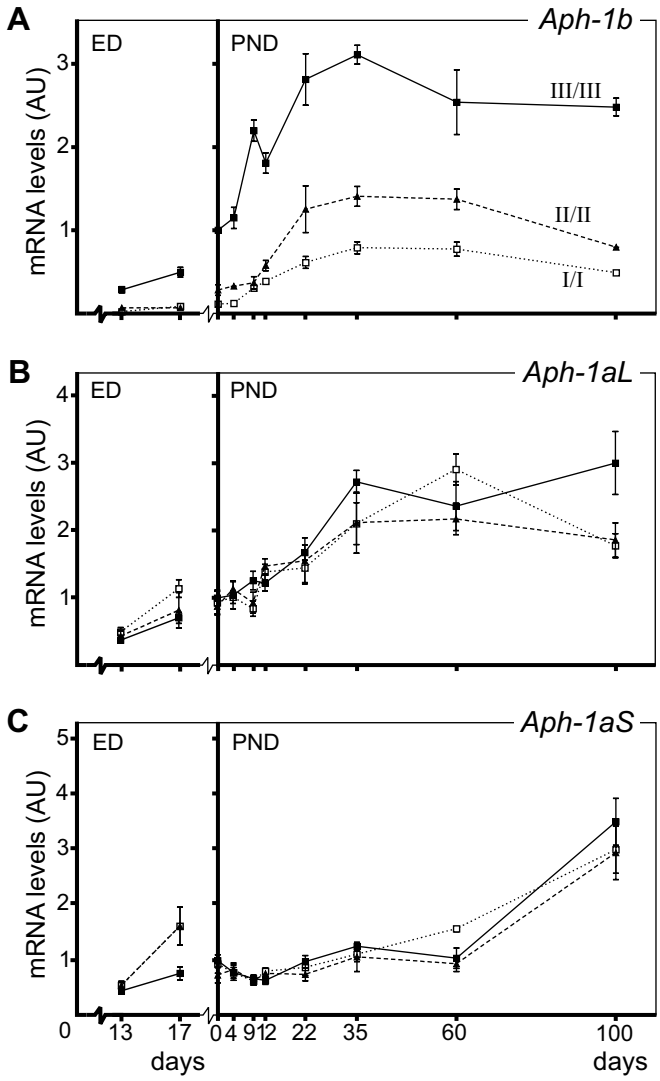

Figure 1. Longitudinal study on *Aph-1aL*, *-1aS* and *-1b* mRNA expression levels in the I/I, II/II and III/III rats

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 normalised 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.

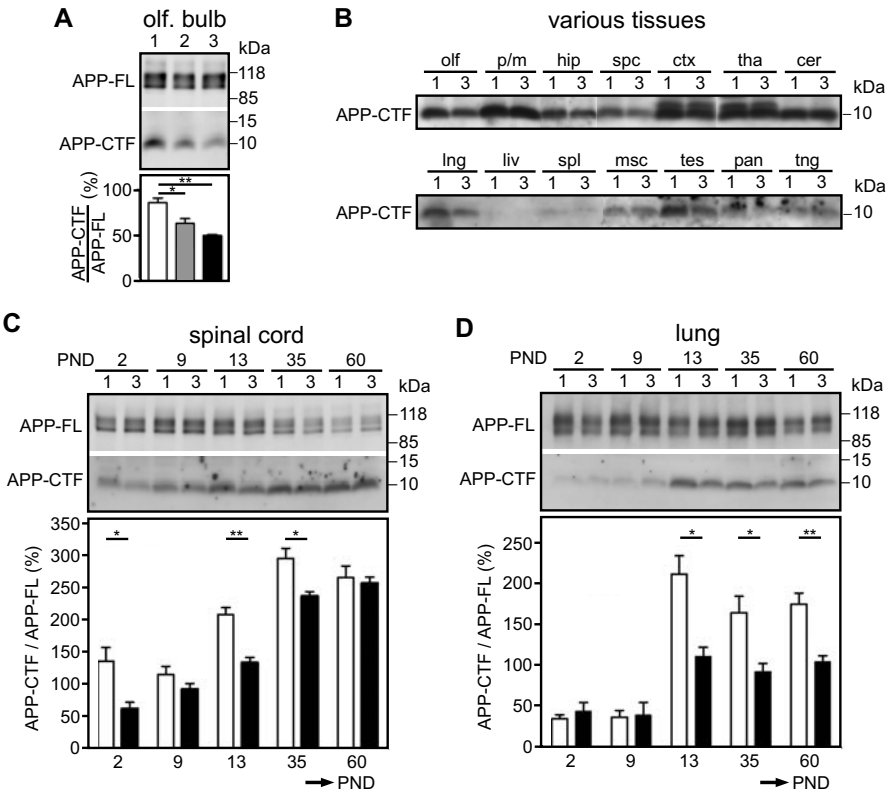

Figure 2. Ontogenic analysis of gamma-secretase cleavage activity towards APP in the I/I, II/II and III/III rats

(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 CTF_{β}(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.).

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.

Discussion

The γ-secretase enzyme is involved in a large variety of developmental signalling pathways (Artavanis-Tsakonas et al., 1999; Huang et al., 2000; Kopan and Ilagan, 2004; Turner et al., 2003). 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/b'* 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 (Saito and Araki, 2005; Serneels et al., 2005; Shirotani et al., 2004). 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; Hasegawa et al., 2004; Li et al., 2003). In contrast, ablation of mouse PS2 expression had little effect on the expression levels

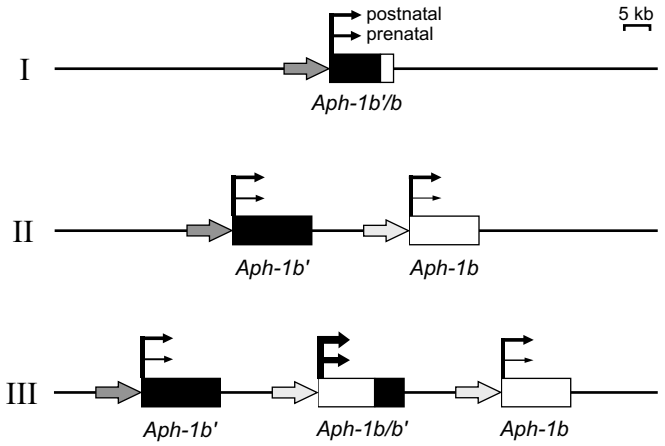

Figure 3. Schematic representation of the *Aph-1b* genes in the I/I, II/II and III/III rat lines

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 (Coolen et al., 2005). 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.

of the other γ-secretase components (Chen et al., 2003; Herreman et al., 1999; 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., 2005). 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 , as the hippocampus is known to modulate the stress axis (Jacobson and Sapolsky, 1991; Knigge, 1961). 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 (Cannon et al., 2002; Isohanni et al., 2000; Jones, 1997). 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.

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) (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., 2005). 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, 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 (Coolen et al., 2005; Ellenbroek and Cools, 2002).

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), 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 $P < 0.05$; 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 5

REDUCED APH-1B EXPRESSION CAUSES TISSUE- AND SUBSTRATE-SPECIFIC CHANGES IN GAMMA-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; Edbauer et al., 2003; Francis et al., 2002; Yu et al., 2000). 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. β-*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'-GTGATTCTCCTCAGTTCTT CCTTAATTC and 5'-GCCCATGAGCACCATGATTATAT; *Aph-1a* (547-670): 5'-AGAGGAGACGGTACTGGGCTTT and 5'-ATGGAAACGGTGACTGCATAGA; β-*actin* (346-435): 5'-CGTGAAAAGATGACCCAGATCA and 5'-AGAGGCATACA-GGGACAACACA; 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).

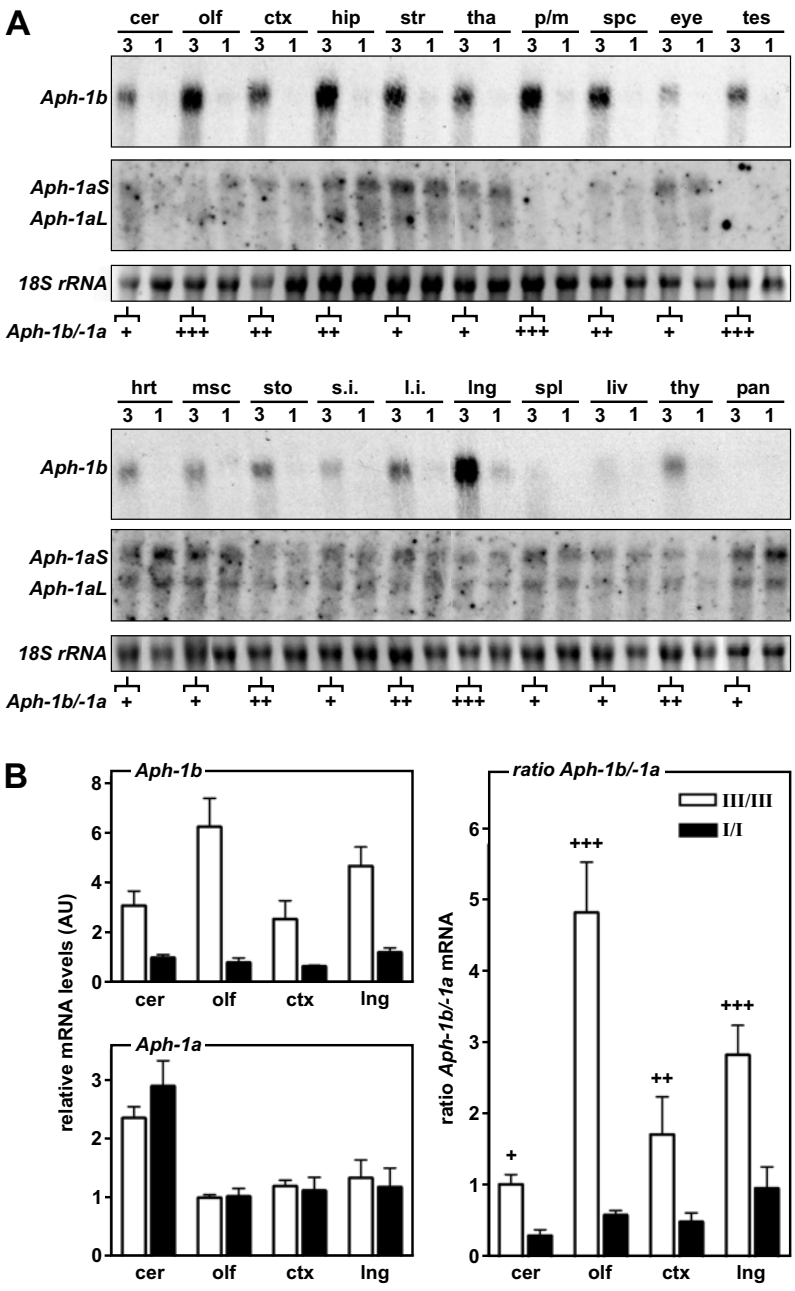
Results

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

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).

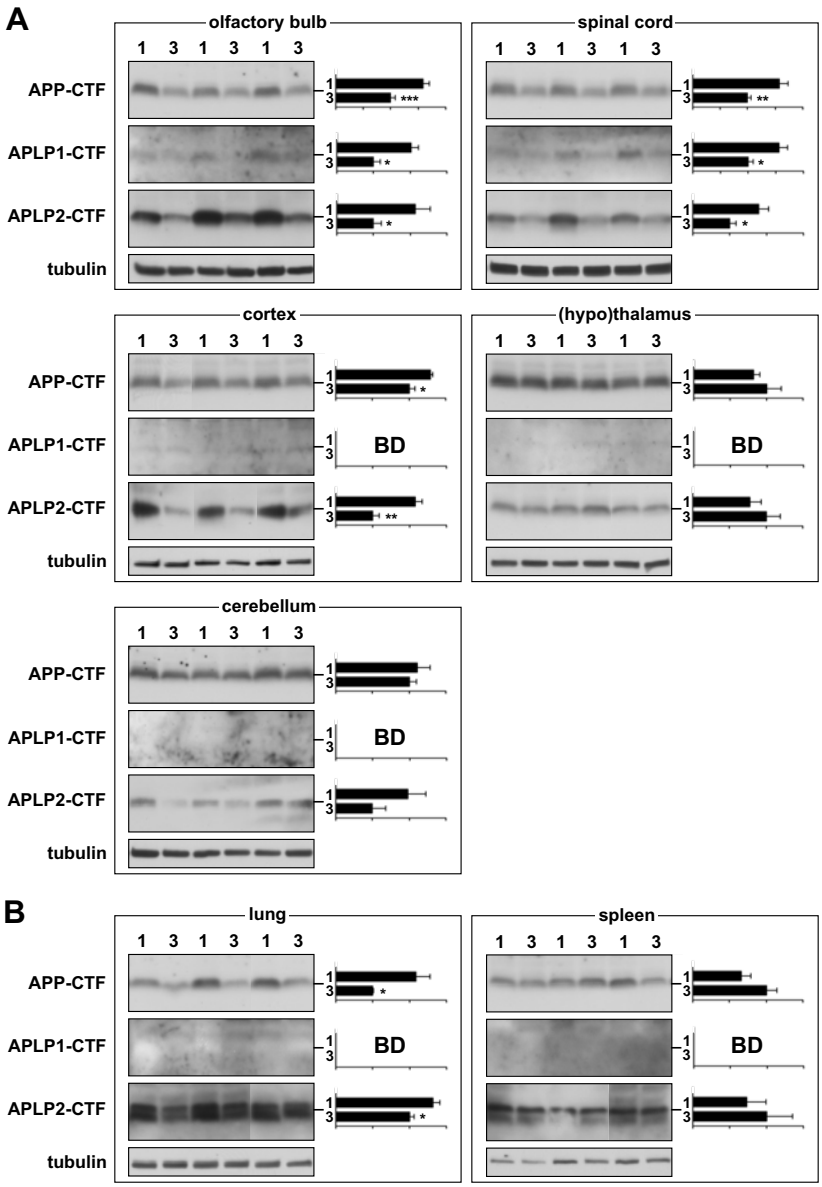
in *Aph-1b* expression. Furthermore, the ratios between the *Aph-1b* and *Aph-1a* mRNA levels greatly varied among the III/III rat tissues. Tissues such as the olfactory bulb, pons/medulla, testis and lung showed high *Aph-1b/-1a* ratios, while the cortex, hippocampus, spinal cord, stomach, large intestine 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).

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

Figure 2. Western blot analysis of the gamma-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.

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).

γ-Secretase cleavage activity towards p75, ErbB4 and NRG2 in I/I and III/III rat tissues

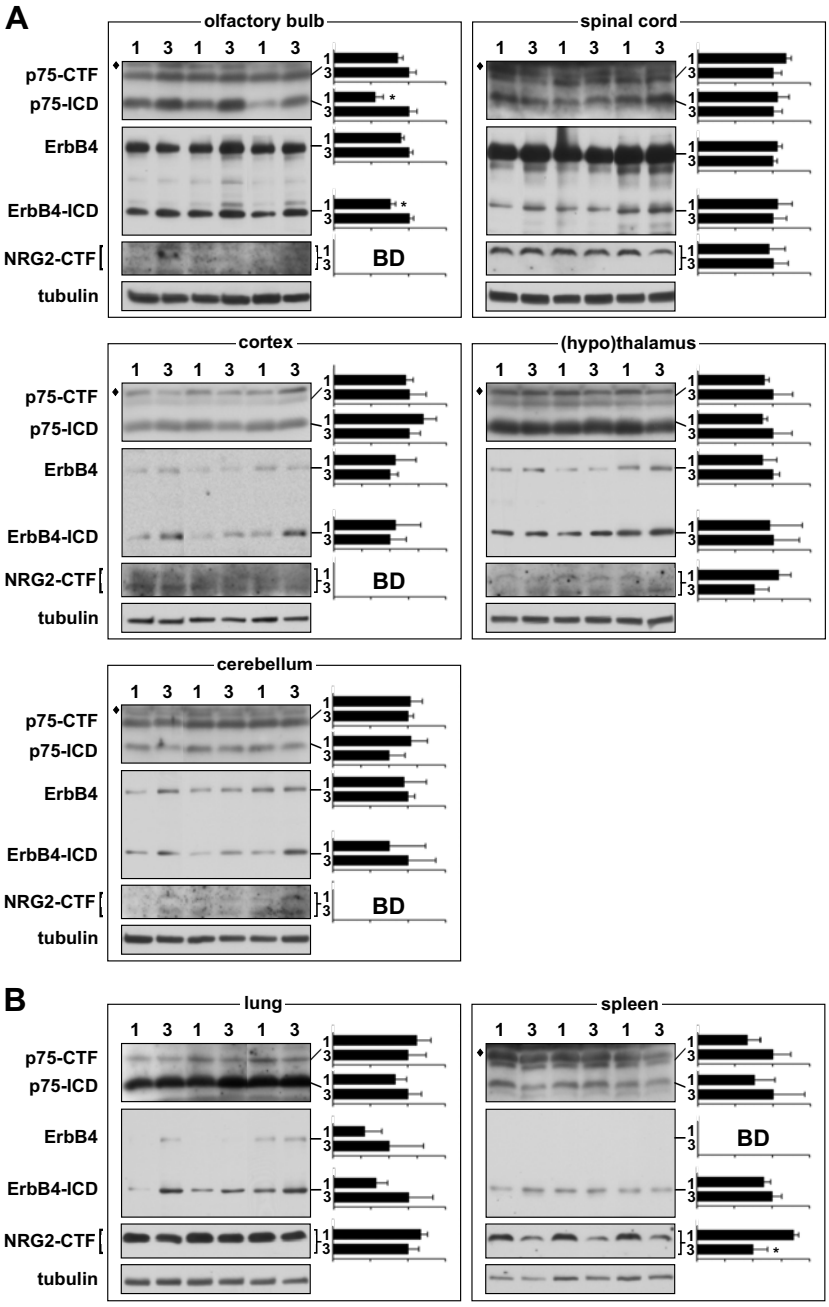
To examine whether substrates other than the APP superfamily members showed affected levels of their γ-secretase cleavage products in I/I compared to III/III rats, we next analysed the cleavages of p75, ErbB4 and NRG2. Although no significant differences were found in the univariate ANOVA for the cleavage 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; Edbauer et al., 2003; Francis et al., 2002; Kim et al., 2003; Lai et al., 2003; Serneels et al., 2005; Yu et al., 2000). 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 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

Figure 3. Western blot analysis of the gamma-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 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 (Coenders et al., 1992; Cools et al., 1990; 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 (Li et al., 2003; Ma et al., 2005; Serneels et al., 2005; Shen et al., 1997), 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 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 (Lee et al., 1996; Page et al., 1996; Thinakaran et al., 1995; 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 Harkin et al., 2003; Kelly et al., 1997). These so-called OBX rats show a number of behavioural changes, such as hyperactivity in an open

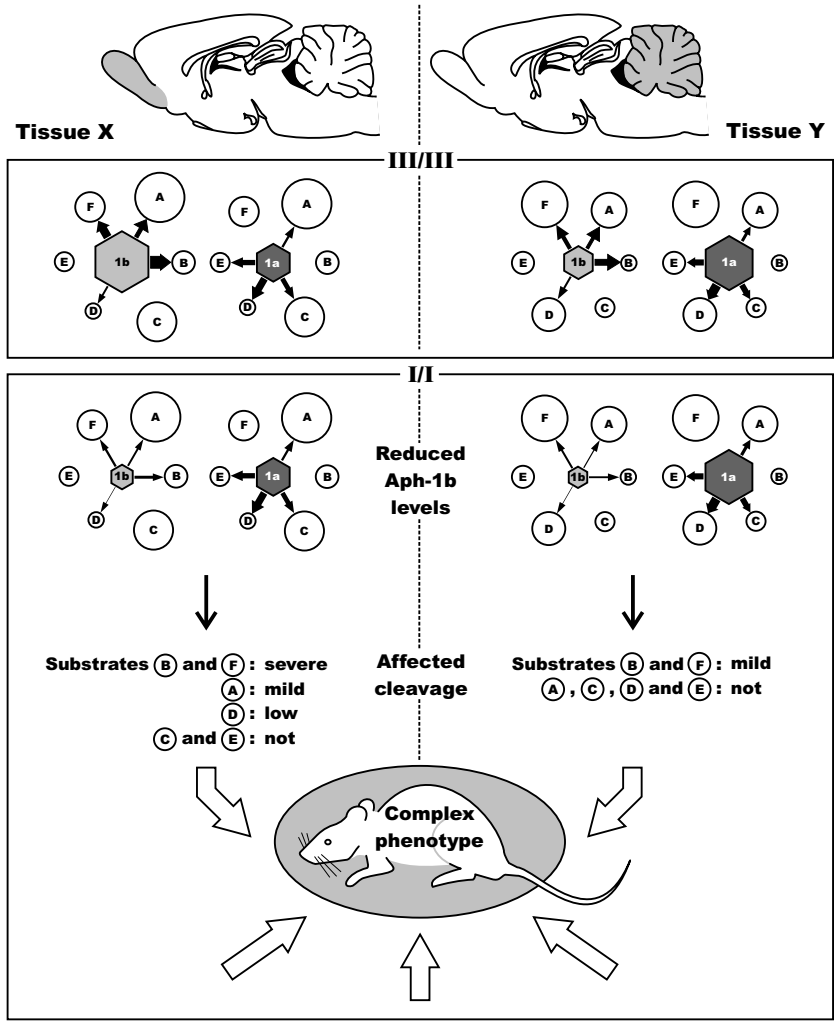


Figure 4. Schematic representation of the tissue- and substrate-specific changes in gamma-secretase activity upon reduced expression of *Aph-1b*, affecting a great variety of signalling pathways and resulting in a complex phenotype

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.

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 (Coolen et al., 2005; Ellenbroek and Cools, 2002). 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 6

TRANSGENE EXPRESSION OF THE GAMMA-SECRETASE COMPONENT APH-1A AFFECTS THE SENSITIVITY OF XENOPUS INTERMEDIATE PITUITARY CELLS TO DOPAMINE

with

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Nick N.H.M. van Bakel,

Gerard J.M. Martens

Abstract

Aph-1 is an essential subunit of the γ-secretase enzyme complex that displays intramembrane catalytic activity towards various type I transmembrane proteins. However, the exact function of Aph-1 remains elusive. Here we investigated *in vivo* the role of one of the Aph-1 family members, Aph-1a, by using the neuroendocrine intermediate pituitary melanotrope cells of the South-African claw-toed frog *Xenopus laevis* as a model system. These cells produce large amounts of the prohormone proopiomelanocortin (POMC) when the animal is placed on a black background, whereby a POMC-derived hormone causes the dispersion of skin melanophores. On a white background the melanotrope cells are inactive because of directly innervating inhibitory neurons of hypothalamic origin, including dopaminergic neurons. Here we employed the technique of stable *Xenopus* transgenesis in combination with a cell-specific POMC gene promoter fragment as a tool to express Aph-1a carboxy-terminally tagged with the green fluorescent protein (Aph1a-GFP) specifically in the melanotrope cells. The Aph1a-GFP fusion protein was synthesised as a ~45-kDa protein and expressed from stage-25 tadpoles onwards, and the expression persisted after metamorphosis. The fusion protein was capable of forming a complex with endogenous presenilin-1. Pulse-chase metabolic cell labelling studies revealed that, despite the high levels of transgene expression, POMC synthesis and processing, and the secretion of POMC-derived products remained unaffected in the transgenic melanotrope cells. Intriguingly, the transgenic melanotrope cells appeared to be more sensitive to the dopamine receptor agonist apomorphine, i.e. their regulated secretion of newly synthesised POMC-derived products was more inhibited than secretion from wild-type cells. Furthermore, the transgenic frogs adapted more rapidly to a white background than wild-type animals. Together, the results of our preliminary functional analysis suggest that transgene expression of Aph-1 in *Xenopus* melanotrope cells leads to a modified sensitivity of the dopaminergic system, in line with the differential expression of Aph-1b in the APOSUS and -UNSUS rats that display different susceptibilities to apomorphine.

Introduction

The γ-secretase enzyme complex is notoriously known for its pathological involvement in Alzheimer's disease. Under physiological conditions the γ-secretase complex is responsible for the intramembrane cleavage of a variety of type I transmembrane proteins, including amyloid-β precursor protein APP, Notch and neuregulin. Active γ-secretase is a tetrameric protein complex consisting of presenilin-1 (or -2), nicastrin, PEN-2, and Aph-1. In mammals, three homologues of Aph-1 are found, termed Aph-1aS, -1aL and -1b. The γ-secretase complex is found throughout the vertebrates and invertebrate kingdom, from human to silk worm and plant (for a review, see Kimberly and Wolfe, 2003). Recently, we identified a gene-dosage effect of Aph-1b in a rat model with a complex phenotype, whereby the reduced expression of the gene caused many features of a neurodevelopmental disorder such as schizophrenia (Coolen et al., 2005). An alignment of the *Rattus norvegicus* Aph-1aS, -1aL and -1b amino acid sequences, and the Aph-1 sequence of the South-African claw-toed frog

Xenopus laevis revealed a high degree of conservation of the Aph-1 family members, with the rat Aph-1aS sequence being the closest relative of the frog sequence (see Figure 2 of general discussion). To gain more insight into the function of Aph-1, we here used stable *Xenopus* transgenesis for *in vivo* overexpression in the intermediate pituitary melanotrope cells. Since at the time of the generation of the transgenic frogs, the full-length *Xenopus laevis* Aph-1 coding sequence was not known, we decided to use the rat Aph-1aS sequence carboxy-terminally tagged with the green fluorescent protein GFP for transgene expression.

The *Xenopus* melanotrope cells constitute a homogeneous population of strictly regulated neuroendocrine secretory cells. In the melanotrope cells, the prohormone proopiomelanocortin (POMC) is processed to a number of bioactive peptides, including α-melanophore stimulating hormone (α-MSH), a peptide that stimulates pigment dispersion in dermal melanophores, thus causing skin darkening (Jenks et al., 1977). *Xenopus* has thus the unique capability of adapting its skin colour to the background (dark or light). Adaptation to a black background involves a stimulation of α-MSH release, while in animals on a white background the release of α-MSH is inhibited, and consequently, the skin colour becomes lighter. The regulation of α-MSH release is conducted from various brain regions by neuropeptides and neurotransmitters, including dopamine (Kolk et al., 2002; Roubos et al., 2005). POMC is the major cargo protein in the melanotrope cells and during adaptation to a black background the amount of POMC mRNA is induced 30-fold, and cell activity and cell size increase enormously (for a review, see Roubos, 1997). Placing the amphibian on a white or a black background thus allows physiological manipulation of the biosynthetic and secretory activity of the melanotrope cell.

In this study, we combined the unique properties of the melanotrope cell with the technique of stable *Xenopus* transgenesis (Kroll and Amaya, 1996; Sparrow et al., 2000) to drive transgene expression of Aph-1aS in a cell-specific manner. A DNA construct was made that encodes *Rattus norvegicus* Aph-1aS fused to the amino-terminus of GFP and under the control of a *Xenopus* POMC gene A promoter fragment that directs expression of the fusion protein specifically to the *Xenopus* melanotrope cells, leaving the integrity of the regulation by the hypothalamic neurons intact. The results indicate that in the generated *Xenopus* transgenic for Aph-1aS-GFP the melanotrope cells overexpress the fusion protein and have a more sensitive dopaminergic system than wild-type cells.

Materials and Methods

Animals

South-African claw-toed frogs *Xenopus* laevis were bred and reared at the Central Animal Facility of the Radboud University of Nijmegen. For the transgenesis experiments, female *Xenopi* were directly obtained from South Africa (Xenopus Express, Cape Town, South-Africa). Animals were adapted to a black or white background under constant illumination at 28°C for at least three weeks. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and permit TRC 99/15072 to generate and house transgenic Xenopus.

Quantitative RT-PCR

For quantitative RT-PCR, neurointermediate lobes (NILs) and anterior lobes (ALs) of black- and white-adapted Xenopus pituitaries were dissected and total RNA was isolated using the Trizol method. First-strand cDNA was prepared from 2 µg of DNase I treated total RNA 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. *GAPDH* 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: X-*APH1-RQ*-FW: 5'-CAGTACGGGCTCTGGATATTTGG-3', X-*APH1-RQ*-RV: 5'-CGGTCCTCACTGATCGTAGCA-3', X-*GAPDH-RQ*-FW: 5'-GCCGTGTATGTGGTGGAATCT-3' and X-*GAPDH-RQ*-RV: 5'- AAGTTGTCGTT-GATGACCTTTGC-3'.

Antibodies

Polyclonal antibody ST62 directed against the precursor form of *Xenopus* POMC (Berghs et al., 1997), polyclonal antibody C87 raised against the carboxy-terminal 12 amino acids of *Xenopus* APP (Collin et al., 2005), polyclonal antibody PS1 against the carboxy-terminal fragment of *Xenopus* PS1 (Tsujimura et al., 1997), polyclonal antibody anti-PC2 against recombinant mature human PC2 (Van Horssen et al., 1998), polyclonal antibody anti-1262CH against the carboxy-terminal region of *Xenopus* p24$\delta_{1/2}$ (Kuiper et al., 2001) and monoclonal anti-tubulin antibody E7 (Chu and Klymkowsky, 1989) have been described previously. A polyclonal antiserum against GFP was kindly provided by Dr. B. Wieringa (Cuppen et al., 1999). Antibodies against Nct (Herreman et al., 2003), PEN-2 and Aph-1a (Nyabi et al., 2003) were tested on lysates from *Xenopus* tissues via western blotting, but did not yield any specific results.

Generation of the DNA constructs encoding Aph-1aS fused to the amino-terminus of GFP (Aph1aS-GFP)

To generate the Aph1aS-GFP DNA construct, Aph-1aS cDNA was prepared by RT-PCR on RNA from Wistar rat brain using primers based on the *Rattus norvegicus* Aph-1aS coding sequence. The coding sequence of Aph-1aS lacking the stop codon was amplified using primers containing *Eco*RI and *Sal*I restriction sites at their 5' ends (*Rn*Aph-1aS-*Eco*RI-FW: 5'-GGTTGAATTCCCCAGCTGTCCAGTCATG-3' and *Rn*Aph-1aS-*Sal*I-RV: 5'-GGTTGTCGACGTCCTTACACGAAAGGCTGCG-3', respectively, with the restriction sites underlined) resulting in an 820-bp PCR product. Inserting the *Eco*RI×*Sal*I-digested PCR fragments into the pEGFP-N3delAUG vector (Clontech, Mountain View, CA, USA) generated the pEGFP-N3delAUG-Aph-1aS construct with the GFP fusion construct under the control of the CMV promoter. The construct was verified by cycle

DNA sequencing using the Big Dye Ready Reaction system (Applied Biosystems, Foster City, CA) and the primers CMV-IE-FW (5'- GCCTAATGGGAGGTCTATATAAGC-3') and GFP-RV (5'-TTTACGTCGCCGTCCAGCTC-3') that allowed sequence analysis into the Aph-1aS DNA sequence from the CMV promoter sequence and the GFP sequence, respectively. This construct was used for transfection studies in COS-1 (African green monkey kidney) and Neuro2A (mouse neuroblastoma) cells (see below). The Aph1aS-GFP fragment of pEGFP-N3delAUG-Aph-1aS was subcloned into the pPOMC(A)2+ vector (Jansen et al., 2002). To this end, pEGFP-N3delAUG-Aph-1aS was digested with *Not*I, blunted and digested with *Eco*RI, and the 1579-bp Aph1aS-GFP fragment was inserted into the *Eco*RI×*Stu*I-digested pPOMC(A)2+ vector, giving rise to the pPOMC-Aph1aS-GFP construct.

Transfection studies

COS-1 or Neuro 2A cells were transfected with the pEGFP-N3delAUG-Aph-1aS construct using Fugene 6 transfection reagent (Roche, Basel, Switzerland) and after 24 hours, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, enclosed with Mowiol (200 µg/µl), and localisation of the GFP signal was analysed under a Leica DMRA fluorescent microscope and photographs were taken with a Cohu high-performance charge-coupled device camera using the Leica Q Fluoro software.

Preparation of Xenopus unfertilised eggs

To harvest unfertilised eggs, mature female *Xenopus laevis* were injected with 375 IU human gonadotropic hormone (Pregnyl; Organon, Oss, The Netherlands) into their dorsal lymphatic cavities. Eggs were collected from the females 18 hr following injection, de-jellied in 2% cysteine/1x MMR (100 mM NaCl, 1 mM KCl, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5 mM Hepes; pH 8.2), extensively washed with 1×MMR, put in 0.4x MMR/6% Ficoll-400 with 50 µg/ml gentamycin, and immediately used for transgenesis.

Generation of Xenopus transgenic for Aph1aS-GFP

For the generation of transgenic *Xenopus*, a *Bss*HII/*Not*I fragment of about 2600 bp, containing the pPOMC-Aph1aS-GFP construct followed by the SV40 polyA signal, was purified using a Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA fragment (~150 ng/µl) was mixed with sperm nuclei ($2.5*10^5$ in 2.5 µl), incubated for 15 min at room temperature, and diluted to 500 µl. About 10 nl was injected per egg. Sperm nuclei were prepared as described previously (Jansen et al., 2002; Sparrow et al., 2000). Normally cleaving embryos were selected at the four-cell stage and cultured in 0.1x MMR/6% Ficoll-400 with 50 µg/ml gentamycin at 18°C until gastrulation (stage 12) was reached. At that time point, embryo culturing was continued in 0.1× MMR with 50 µg/µl gentamycin at 22°C. From stage 45 onwards, tadpoles were raised in tap water at 22°C. The presence of GFP fluorescence was examined in living embryos anaesthetised with 0.25 mg/ml MS222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO) using

a Leica MZ FLIII fluorescent stereomicroscope and photographs were taken with a Leica DC200 colour camera using the Leica DC viewer software. Staging of *Xenopus* embryos was carried out according to Nieuwkoop and Faber (1967).

In vitro fertilisation of wild-type Xenopus eggs with transgenic sperm cells

For in vitro fertilisation, the testis of a male transgenic *Xenopus* frog was isolated and gently pulled apart prior to use. Pieces of testes were rubbed against unfertilised eggs harvested from wild-type *Xenopus* females. After 10 min, the eggs were incubated in 0.1× MMR. The fertilised eggs were selected in their 4-cell stage and screened for specific pituitary fluorescence during early development (stage 45). Remaining pieces of testes were used to isolate sperm nuclei that were stored at -80°C for future injection experiments to obtain additional transgenic animals.

Background adaptation

For black-to-white background adaptation, wild-type and transgenic animals fully adapted to a black background (at least two months) were placed individually on a white background under constant illumination and the dermal melanophores of the webs were analysed for their dispersion state at fixed time points. The dispersion state was indexed from completely aggregated around the nucleus (melanophore index 1) to maximal dispersed throughout the cytoplasm (index 5; Figure 6A). Wild-type (wt) and transgenic (tr) animals were transferred individually to white buckets by an independent person, who labelled the buckets either 'wt' or 'tr' on the bottom side, invisible to the experimenter (a blinded test set-up). The melanophore index was determined by the experimenter at the set time intervals and following index scoring, the 'wt' and 'tr' labels were combined with the measurements. For white-to-black background adaptation, animals that were adapted to a white background for at least two months were placed on a black background and indexed as in the black-to-white background adaptation experiment.

Western blot analysis

For western blot analysis, NILs were homogenised in TTD buffer (50 mM Hepes, 140 mM NaCl, 0.1% Triton X-100, 1% Tween-20, 0.1% deoxycholate, 1 mM EDTA, 1 mM PMSF, and 0.1 mg/ml soybean trypsin inhibitor; pH 7.3). After the lysates were cleared by centrifugation at 13,000 x g at 4°C, they were denatured in Laemmli sample buffer at 100°C for 5 min, separated on 12.5% SDS-PAGE, and transferred to nitrocellulose (for GFP, APP, POMC, PC2 and p24δ) or PVDF (for PS1) membranes. For extraction under native conditions, NILs were first homogenised in CHAPS buffer (1% CHAPS, 5 mM EDTA, 1 mM PMSF, and 0.1 mg/ml soybean trypsin inhibitor in 1x PBS) followed by centrifugation at 1000 x g for 10 min at 4°C yielding a post-nuclear supernatant. Following blocking in 5% skimmed milk/1% Tween-20/PBS for one hr, blots were incubated with anti-GFP (1:5000), anti-PS1 (αPS1; 1:2000), anti-

APP (C87; 1:3000), anti-POMC (ST62; 1:20,000), anti-PC2 (hPC2; 1:5000) and anti-p24$\delta_{1/2}$ (anti-1262CH; 1:5000) and anti-tubulin (E7; 1:500) overnight at 4°C. After extensively washing with 1% skimmed milk/1% Tween-20/PBS for 30 min at RT, blots were incubated with a peroxidase conjugated secondary antibody (1:5000) for 45 min at RT, and subsequently thoroughly washed with 1% skimmed milk/1% Tween-20/PBS for 30 min at RT. Proteins on western blots were immunodetected using Lumi-Light substrate (Roche Diagnostics, Mannheim, Germany) and subsequently exposed to X-ray film (Kodak, Rochester, NY). Quantification was performed using a BioChemi imaging system and signals were analysed using Labworks 4.0 software (UVP BioImaging systems, Cambridge, UK).

Co-immunoprecipitation analysis

For co-immunoprecipitation analysis, NILs were lysed in CHAPS buffer (1% CHAPS, 5 mM EDTA, 1 mM PMSF, and 0.1 mg/ml soybean trypsin inhibitor in 1x PBS) and the extract was diluted with TTD buffer to 1 ml, and supplemented with SDS (final concentration of 0.08%) and an α-GFP antibody (1:500). Precipitation was performed O/N at 4°C while rotating the samples. Immune complexes were precipitated with protein A-sepharose (Amersham Biosciences) for 6 hr at 4°C while rotating the samples and resolved by SDS-PAGE. After transfer to PVDF membrane, the blot was incubated with anti-PS1 as described under western blot analysis.

Metabolic cell labelling

For metabolic cell labelling, NILs of black-adapted wild-type and transgenic *Xenopus* were rapidly dissected and preincubated in Ringer's medium for 15 min at 22°C. Radioactive labelling of newly synthesised proteins was performed by incubating the NILs in Ringer's medium containing 5 mCi/ml Tran^{35}S-label for 30 min at 22°C, rinsed, and chased with 0.5 mM L-methionine in Ringer's medium for three hours. After the chase, NILs were homogenised on ice in TTD buffer and the lysates were cleared by centrifugation at 13,000 x *g* for 7 min at 4°C. Parts of the lysates and incubation media were analysed directly on SDS-PAGE, while the remainder was used for western blot.

Apomorphine treatment

For the analysis of the influence of apomorphine on POMC processing and release of the POMC-derived peptides, dissected wild-type or transgenic NILs were pulsed and chased as described above, whereby apomorphine was added during the chase period to a final concentration of $2.0*10^{-8}$ M or $6.7*10^{-9}$ M.

Statistics

Data from the quantitative RT-PCR and background adaptation experiments are presented as means ± s.e.m. (n = 3 and n = 8 per group, respectively). Statistical evaluation of the RT-PCR data was performed using an unpaired Student's *t*-test, while

the background adaptation experimental data were analysed a univariate ANOVA on the melanophore index of the webs of wild-type and transgenic frogs. Since the univariate ANOVA yielded statistical significance, we performed an independent samples T-test over the melanophore index for every time point, and for both wild-type and transgenic frogs a one-way ANOVA and post hoc a Bonferroni analysis. Differences are significant if $P < 0.05$.

Results

Endogenous Aph-1 mRNA is not differentially expressed in the pituitary of black- and white- adapted Xenopus laevis

In *Xenopus*, the *Aph-1* gene family consists of only one gene, while mammals have two *Aph-1* genes (*Aph-1a* and *Aph-1b*). To determine the levels of endogenous *Xenopus Aph-1* transcripts in the NIL and AL of the pituitary of animals adapted to a black or white background, we performed real-time quantitative RT-PCR analysis. The melanotrope cells in the NIL regulate the process of background adaptation. Depending on the colour of the background of the frog, these cells are either highly activated or inhibited by neurons of hypothalamic origin. In contrast, the anterior pituitary cells are not involved in background adaptation (Martens et al., 1987). In both NIL and AL, *Aph-1* mRNA was detected, but no significant differences were observed between the levels in the NILs of black- and white-adapted animals, nor between the levels in the ALs (Figure 1). Despite many attempts to study the expression levels of *Xenopus* Aph-1 at the protein level, none of the available antibodies recognised a specific product in the NIL or AL.

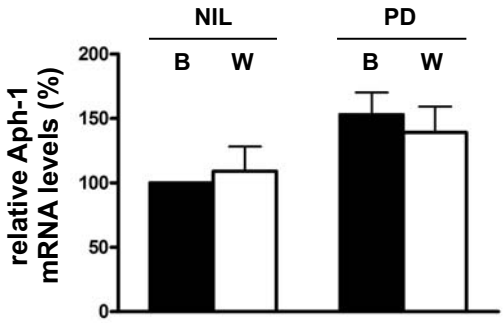

Figure 1. Real-time quantitative RT-PCR analysis of endogenous *Aph-1* mRNA levels in the neurointermediate lobe (NIL) and anterior lobe (AL) of the pituitary of black- (B) and white- (W) adapted *Xenopus*

The level of *Aph-1* mRNA in the NIL of black-adapted animals was set to 100%. No differences in *Aph-1* mRNA levels were observed in the NILs or ALs of black- and white-adapted animals. In each case, the values shown are relative to the amounts of *GAPDH* mRNAs and represent the mean plus SEM from 3 different animals.

Generation of Xenopus transgenic for the Aph1aS-GFP fusion protein

The *Xenopus* Aph-1 sequence is most highly related to the mammalian Aph-1a sequence (see Figure 2 of the General Discussion). To study functional aspects of Aph-1, we used the short form of rat Aph-1a (Aph-1aS) to produce a transgenesis DNA construct and then generated *Xenopus* transgenic for Aph-1aS fused to the amino-terminus of GFP (Aph1aS-GFP). The DNA construct containing the sequence encoding Aph1aS-GFP downstream of the CMV promoter (pEGFP-N3delAUG-Aph-1aS; Figure 2A) was first used for transfection studies in COS-1 and Neuro2A cells. Twenty-four hours after transfection the Aph1aS-GFP cells revealed fluorescence in a network-like structure throughout the cytoplasm, but predominantly at one side of the nucleus (Figure 2B). Western blot analysis of transfected COS1 cell lysates showed that the Aph1a-GFP fusion protein migrated as a ~45-kDa protein, whereas an additional product of ~85 kDa and high-molecular weight products of >110 kDa were observed as well (Figure 2C). Similar results were obtained when lysates of transfected Neuro2A cells were analysed (data not shown). In these analyses, a denaturing lysis buffer was used and the samples were heated to 100°C prior to loading, which is known to cause co-aggulation of multi-transmembrane proteins (Hansson et al., 2005; Shirotani et al., 2004), eventually resulting in the observed high-molecular weight products.

Subcloning of the Aph-1aS insert from the pEGFP-N3delAUG-Aph-1aS construct into the pPOMC(A)2+ vector generated a construct with the insert downstream of a 529-bp *Xenopus* POMC gene A promoter fragment (pPOMC-Aph-1aS-GFP), which directs transgene expression specifically to the melanotrope cells of the *Xenopus* intermediate pituitary (Jansen et al., 2002). The linearised pPOMC-Aph-1aS-GFP DNA (Figure 3A) was mixed with *Xenopus* sperm nuclei from a male wild-type animal, the mixture was microinjected into unfertilised *Xenopus* eggs from a female wild-type animal and a number of fluorescent tadpoles were generated (Figure 3B). The different levels of Aph1aS-GFP expression among the various F_0 transgenic animals could be readily and directly established by visual inspection of the living *Xenopus* tadpoles under a fluorescence microscope. The expression of the Aph1aS-GFP fusion protein was restricted to the intermediate lobe (IL; neuroendocrine melanotrope cells) of the pituitary, while the pituitary AL, in which the POMC producing corticotrope cells are located, and other brain structures did not show any fluorescence (Figure 3C). An F_1 offspring was generated by *in vitro* fertilisation of eggs harvested from wild-type *Xenopus* females with sperm isolated from the testis of a male *Xenopus* frog transgenic for pPOMC-Aph1aS-GFP. We selected a transgenic F_1 line (#2) of which all offspring showed relatively high Aph1aS-GFP transgene expression and raised these embryos for further analysis.

Steady-state levels of the Aph1aS-GFP fusion protein, POMC, PC2 and p24$\delta_{1/2}$ in the pituitary of Xenopus transgenic for Aph1aS-GFP

From the adult *Xenopus* pituitary (consisting of the pars nervosa, IL and AL), the AL can be dissected, but the pars nervosa (containing nerve terminals of hypothalamic origin) is intimately associated with the IL. To examine the steady-state levels of Aph1aS-GFP protein expression, the NIL was dissected from black-adapted frogs transgenic for

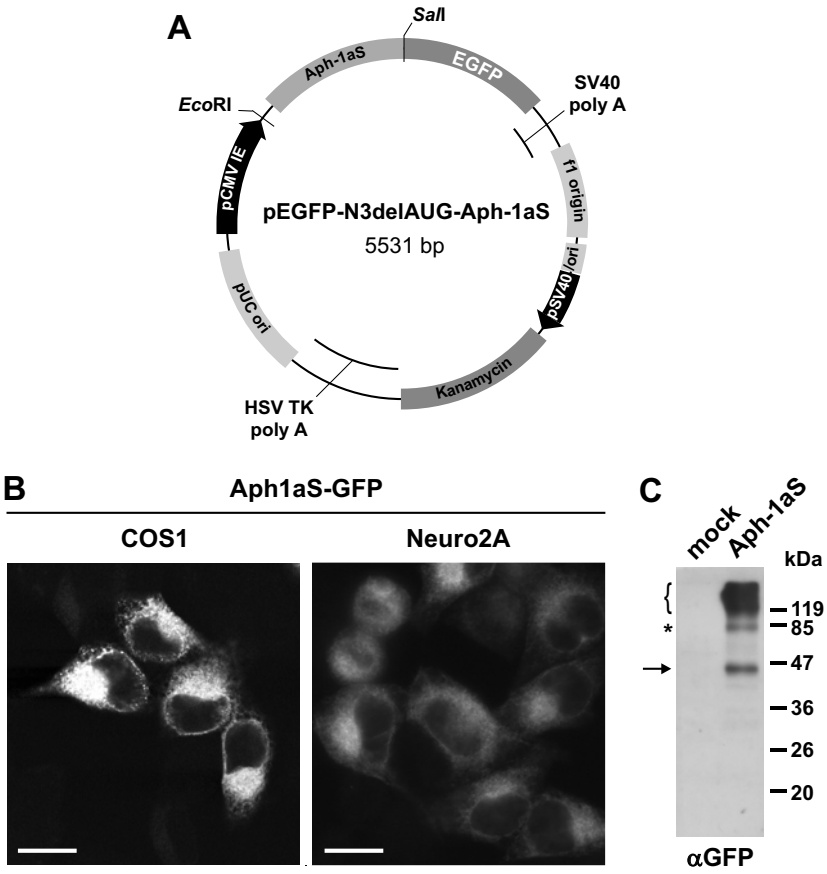

Figure 2. Expression of the Aph1aS-GFP fusion protein in transfected COS1 and Neuro2A cells

(A) Schematic representation of the circular construct pEGFP-N3delAUG-Aph-1aS containing the *Rattus norvegicus* Aph-1aS coding sequence fused to the amino-terminus of GFP. The fusion construct was under the control of the CMV promoter and the selection marker gene *kanamycin* was under the control of the SV40 promoter; SV40 poly A and HSV TK poly A, polyadenylation signals; /ori (upstream of pSV40), SV40 origin for replication in mammalian cells expressing the SV40 T-antigen; F1 origin, origin for single-stranded DNA production; pUC ori, origin for replication in *E. coli*. (B) Fluorescence microscopy on COS1 cells (left panel) and Neuro2A cells (right panel) transfected with pEGFP-N3delAUG-Aph-1aS. Direct GFP fluorescence was observed in a network-like structure throughout the cytoplasm, but most prominent near the nucleus. Bars equal 25 μm. (C) Western blot analysis of proteins extracted from COS1 cells transfected with pEGFP-N3delAUG-Aph-1aS (Aph1aS-GFP) or mock transfected. In addition to a number of higher molecular weight products ({: ≥119 kDa; *: ~85 kDa in size), the anti-GFP antibody staining revealed a product of ~45 kDa (indicated by the arrow) in the Aph1aS-GFP-transfected cell lysate. No product was detected in the lysate of the mock-transfected cells.

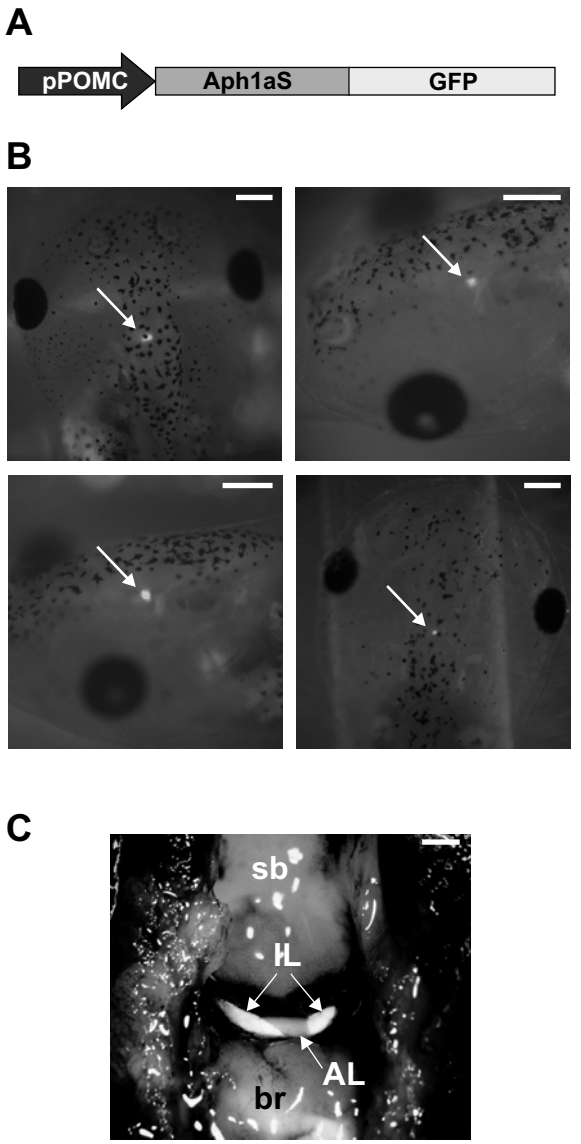

Figure 3. Intermediate pituitary-specific fluorescence in *Xenopus* embryos transgenic for Aph1aS-GFP

(A) Schematic representation of the linear injection fragment pPOMC-Aph1aS-GFP containing the *Xenopus* POMC gene A promoter fragment (pPOMC) and the Aph1aS-GFP fusion protein-coding sequence that was used to generate transgenic *Xenopus*. (B) Intermediate-pituitary-specific fluorescence in living *Xenopus* embryos (stage ~40) transgenic for the Aph1aS-GFP fusion protein. Arrows indicate the localisation of the fluorescent intermediate pituitary expressing the fusion product. Bars equal 0.5 mm. (C) Ventrocaudal view on the brain of a black-adapted 6 month-old frog transgenic for Aph1aS-GFP. The brain (br) was lifted to reveal intense fluorescence in the intermediate lobe (IL), but not in the anterior lobe (AL) of the pituitary; sb, skull base. Bar equals 1 mm.

Aph1aS-GFP and wild-type animals, and homogenised in CHAPS buffer. Western blot analysis of the NIL lysates from the transgenic animals revealed that the majority of the Aph1aS-GFP fusion protein migrated as a ~45-kDa protein, whereas an additional higher molecular weight band of ~90 kDa was observed. No GFP signal was observed in lysates of the NILs of wild-type animals, or in the ALs of wild-type and transgenic animals (Figure 4A and data not shown). The steady-state protein levels of POMC, the POMC cleavage enzyme PC2 and the putative ER-to-Golgi cargo receptor proteins $p24\delta_{1/2}$ were similar in the NILs and ALs of transgenic and wild-type animals (Figure 4B). In conclusion, expression of the Aph1aS-GFP fusion protein was restricted to the intermediate pituitary melanotrope cells and its level does not appear to affect the steady-state levels of POMC, PC2 or $p24\delta_{1/2}$.

Analysis of the γ-secretase component presenilin-1 and γ-secretase cleavage activity towards APP in the intermediate pituitary cells of Xenopus transgenic for Aph1aS-GFP

Next, we were interested in the effects of the overexpression of the Aph1aS-GFP fusion protein on the expression of the other γ-secretase components in the NILs of the transgenic animals. The γ-secretase complex is ubiquitously expressed and consists of one of the presenilin proteins (PS1 or PS2), Nct, Aph1 and PEN-2. Unfortunately, except for PS1 (Tsujimura et al., 1997), no suitable antibodies were available to detect the endogenous levels of any of these proteins in *Xenopus*. Western blot analysis using the anti-*Xenopus* PS1 antibody revealed no differences between PS1 levels in transgenic and wild-type NIL lysates (Figure 4C, upper panel). To test whether the Aph1aS-GFP fusion protein was associated with endogenous γ-secretase components, we immunoprecipitated NIL proteins with an anti-GFP antibody and subsequently performed western blot analysis of the immunoprecipitate using the anti-PS1 antibody. This co-immunoprecipitation (co-IP) analysis revealed that in the transgenic NIL lysates the carboxy-terminal fragment of PS1 was pulled down with the fusion protein, while no PS1 was detected in NIL lysates from wild-type animals (Figure 4D).

We then decided to determine the effects of the overexpression of Aph1aS-GFP on γ-secretase cleavage activity by analysing one of the best-known substrates of γ-secretase, namely the amyloid-β precursor protein APP. The proteolytic processing of APP starts with shedding of its extracellular domain by α- or β-secretase, leaving a carboxy-terminal fragment (CTF) that is subsequently cleaved by γ-secretase. Western blot analysis showed similar amounts of both the APP holoprotein (APP-FL) and the APP-CTF, indicating no direct effect of the overexpression of Aph1aS-GFP on the proteolytic processing of APP by the γ-secretase complex (Figure 4E).

Biosynthesis and processing of newly synthesised POMC in Xenopus melanotrope cells transgenic for Aph1aS-GFP

We next examined the effect of the overexpressed Aph1aS-GFP fusion protein on the biosynthesis and processing of POMC as well as the secretion of the POMC-derived

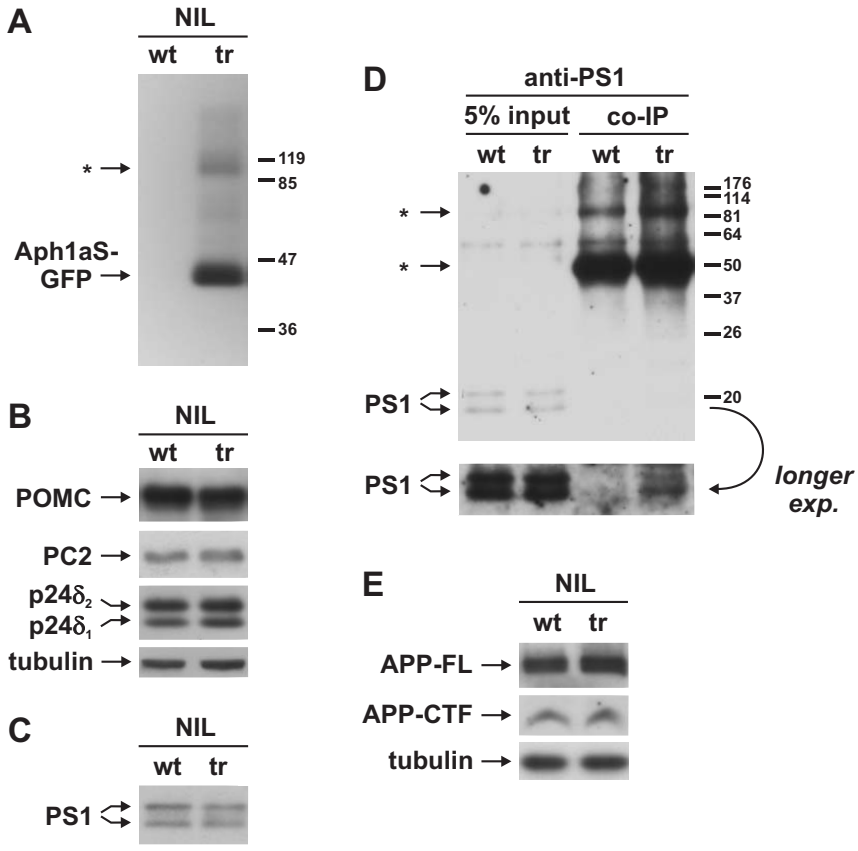

Figure 4. Steady-state levels of the Aph1aS-GFP fusion protein and a number of *Xenopus* intermediate pituitary proteins in the NILs of frogs transgenic for Aph1aS-GFP and wild-type animals

(A) Western blot analysis of native tissue lysates of neurointermediate lobes (NILs) derived from wild-type animals (wt) and frogs transgenic for the Aph1aS-GFP fusion protein (tr) using an anti-GFP antibody (α-GFP). In the transgenic NIL, a prominent product of ~45 kDa and an additional product (*)of ~90 kDa was observed. (B) Western blot analysis of lysates of NILs from wild-type animals (wt) and animals transgenic for Aph1aS-GFP (tr) using anti-POMC, anti-PC2, anti-p24δ1/2, and anti-tubulin antibodies. Tubulin was used as a control for protein loading. (C) Western blot analysis of the γ-secretase component PS1 in transgenic *Xenopus* NILs. Similar amounts of PS1 were detected in the wild-type and transgenic NILs. (D) Co-immunoprecipitation (co-IP) analysis of NIL lysates from wild-type animals (wt) and frogs transgenic for the Aph1aS-GFP fusion protein (tr) using an anti-GFP antibody for immunoprecipitation and an anti-PS1 antibody for western blot analysis. Products of ~18 and ~21 kDa were detected in the transgenic NIL samples and suggested to represent different post-translational modifications of the CTF of PS1. The amounts of PS1-CTF in the input lanes of wild-type and transgenic NILs showed that equal amounts of tissue lysates were used. (E) Western blot analysis of the γ-secretase cleavage activity towards APP in transgenic *Xenopus* NILs. Similar amounts of APP holoprotein (APP-FL) as well as APP C-terminal fragment (APP-CTF) were detected in the wild-type and transgenic NILs, indicating no affected γ-secretase cleavage activity. Tubulin was used as a control for protein loading.

products. To this end, we performed pulse-chase analyses of newly synthesised proteins produced in the tissue and secreted into the incubation medium by NILs of *Xenopus* transgenic for Aph1aS-GFP and wild-type animals. Because besides the melanotrope cells, the *Xenopus* NIL consists of nerve terminals of hypothalamic origin that are biosynthetically inactive (the pars nervosa), the radiolabelled proteins are synthesised by the melanotropes. Following a 30-min pulse labelling and a subsequent three-hour chase, in both wild-type and transgenic NILs most of the 37-kDa POMC precursor protein was processed to an 18-kDa POMC cleavage product, which was subsequently secreted into the incubation medium (Figure 5). The 18-kDa product represents the N-terminal portion of 37-kDa POMC and is generated by the first endoproteolytic cleavage step during POMC processing, while the 14-18-kDa products resemble the small POMC-derived products (Martens, 1986). Aside from a limited reduction in the levels of the secretory products 14-18-kDa POMC and 69-kDa PC2 in the medium, no clear differences were found in the newly synthesised protein levels of transgenic NILs in comparison to wild-type NILs. Together, these results indicate that the transgene expression of the Aph1aS-GFP fusion protein in the intermediate pituitary melanotrope cells had no effect on POMC and PC2 biosynthesis and processing, or on the release of the POMC-derived products.

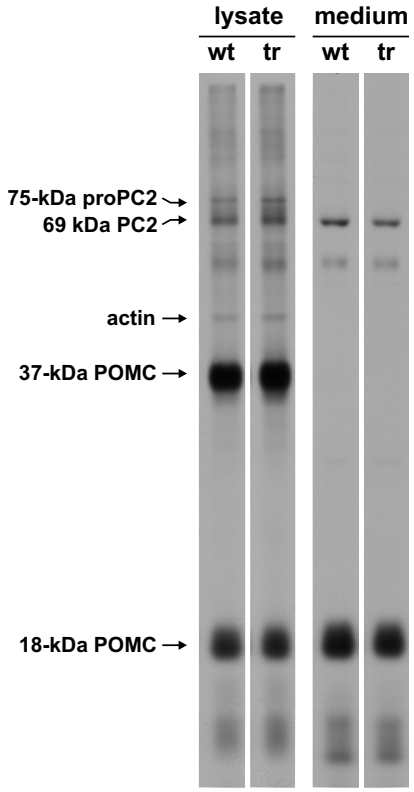

Figure 5. Biosynthesis and processing of newly synthesised POMC in intermediate pituitary cells of *Xenopus* transgenic for Aph1aS-GFP and wild-type animals

Neurointermediate lobes (NILs) of black-adapted wild-type animals (wt) and animals transgenic for Aph1aS-GFP (tr) were pulse labelled for 30 min and subsequently chased for 3 hr. Newly synthesised proteins extracted from the lobes (5%) or secreted into the incubation medium (20%) were resolved by SDS-PAGE on 15% gels and visualised by autoradiography. The experiments were performed in triplicate and a representative example is shown.

Background adaptation of Xenopus transgenic for Aph1aS-GFP

The *Xenopus* intermediate pituitary melanotrope cells are involved in the process of background adaptation (Jenks et al., 1977). We therefore decided to examine the physiological consequence of the expression of the Aph1aS-GFP transgene specifically in the melanotrope cells for background adaptation of the transgenic animal. Upon placing a completely white-adapted *Xenopus* on a black background, the melanotrope cells regulate the dispersion of the melanin in the skin melanophores by releasing α-MSH into the bloodstream resulting in a darkening of the animals skin (Jenks et al., 1977). Simultaneously scoring the melanophore state in the webs of the adapting frogs according to the melanophore index (Figure 6A) allowed real-time analysis of this physiological adaptation process. A white-to black adaptation experiment studying wild-type frogs and frogs transgenic for Aph1aS-GFP did not reveal any significant differences in adaptation speed to a black background (Figure 6B). Surprisingly, in the reverse experiment, fully black-adapted transgenic frogs adapted more rapidly to a white background than wild-type animals, which was significant from t = 0.5 hours onward (Figure 6C; $P < 0.05$).

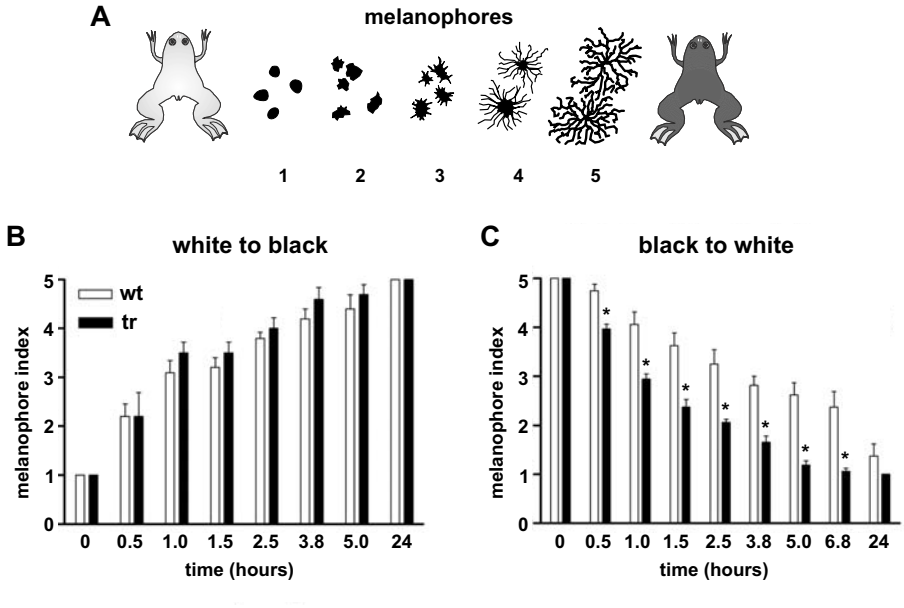

Figure 6. The rate of background adaptation is affected in transgenic *Xenopus* overexpressing Aph1aS-GFP specifically in the intermediate pituitary

(A) Skin melanophore index after Hogben and Slome (1931). The dispersion state of melanin pigment granules in skin melanophores, responsible for the darkening of the skin, was indexed from completely aggregated around the nucleus (melanophore index 1) to maximally dispersed throughout the cytoplasm (index 5). (B) Fully white-adapted wild-type (wt) or transgenic (tr) frogs placed on a black background show similar adaptation rates, based on their melanophore index. Shown are means plus s.e.m. (n = 5). (C) Fully black-adapted transgenic (tr) frogs placed on a white background show a more rapid background adaptation than black-adapted wild-type (wt) animals, based on their melanophore index. Shown are means plus s.e.m. (n = 5).

Effects of apomorphine on the biosynthesis and processing of newly synthesised proteins in Xenopus melanotrope cells transgenic for Aph1aS-GFP

As mentioned above, background adaptation of *Xenopus laevis* is regulated by the amount of α-MSH secreted by the neurointermediate lobe into the blood stream of the frog. Upon placing the animal on a lighter background, α-MSH secretion is reduced, which can be regulated at multiple levels (Roubos et al., 2005). One of these levels is the dopaminergic input from neurons originating in the suprachiasmatic nucleus of the hypothalamus (Tuinhof et al., 1994). Since in the rat a reduced level of Aph-1b affects the dopaminergic system (Coolen et al., 2005), we examined the dopaminergic system in the NILs of wild-type and transgenic animals. To this end, we performed pulse-chase analyses in the presence or absence of various concentrations of the dopaminergic agonist apomorphine. It has previously been found that in the wild-type situation $1*10^{-7}$ M apomorphine caused complete inhibition of secretion of the POMC-derived products into the medium, whereas $2*10^{-8}$ M resulted in ~70% blockage (Eric Jansen, personal communication). We now determined the signal intensities of the 18-kDa POMC in the medium relative to the 37-kDa POMC in the lysate to analyse the level of (inhibition of) regulated secretion in the wild-type and transgenic NILs (Figure 7). Again, in the absence of apomorphine a small reduction in the secretion rate was observed in the transgenic compared to the wild-type NILs, indicated by slightly decreased levels of 18-kDa POMC in the medium relative to 37-kDa POMC in the lysate (compare Figures 5 and 7). Low levels of apomorphine ($7*10^{-9}$ M) clearly decreased the secretion in the transgenic NIL (to ~73%), while the wild-type NIL did not show a difference with the situation in the absence of apomorphine. At levels of $2*10^{-8}$ M apomorphine, both the transgenic and wild-type NILs displayed a clear reduction of secretion of 18-kDa POMC relative to of the presence of $7*10^{-9}$ M apomorphine (Figure 7). Since this experiment has been performed only once, no error bars are presented. However, all three individual NILs of frogs transgenic for Aph1aS-GFP showed a reduced regulated secretion compared to their respective wild-type controls. Therefore, these data indicate that the *Xenopus* melanotrope cells transgenic for Aph1aS-GFP appear to be more sensitive to apomorphine in blocking secretion than wild-type cells.

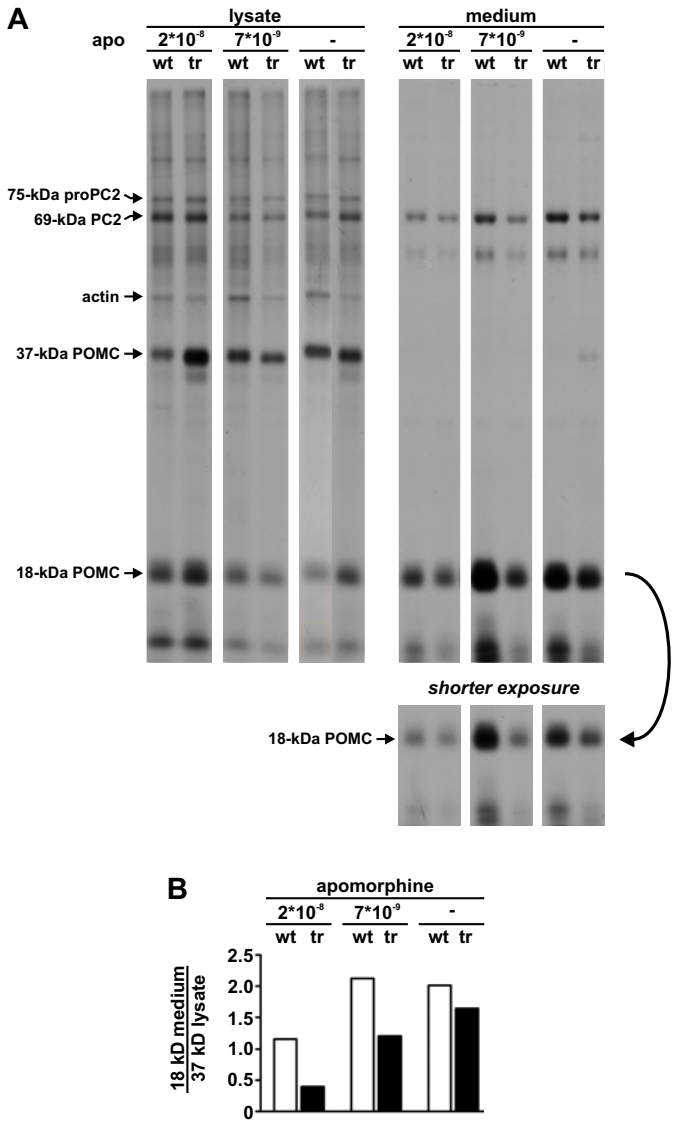

Figure 7. Effect of apomorphine on the biosynthesis and processing of newly synthesised POMC in *Xenopus* intermediate pituitary cells transgenic for Aph1aS-GFP and wild-type cells

(A) Neurointermediate lobes (NILs) of black-adapted wild-type animals (wt) and animals transgenic for Aph1aS-GFP (tr) were pulse labelled for 30 min and subsequently chased for 3 hr in the presence or absence of $2*10^{-8}$ or $7*10^{-9}$ M apomorphine or in chase medium lacking apomorphine (-). Newly synthesised proteins extracted from the lobes (5%) or secreted into the incubation medium (20%) were resolved by SDS-PAGE on 15% gels and visualised by autoradiography. (B) Quantification of the amounts of the 18-kDa POMC product in the medium relative to the levels of the 37-kDa POMC product in the lysates. Similar results were obtained when the levels of 18-kDa POMC product in the medium were normalised to the levels of newly synthesised actin in the lysates (data not shown).

Discussion

The aim of the present study was to study *in vivo* the role of Aph-1, one of the γ-secretase components, by examining the effect of the transgene expression of Aph-1aS in *Xenopus* intermediate pituitary melanotrope cells on prohormone biosynthesis and processing, the secretion of the prohormone-derived peptides, γ-secretase functioning and background adaptation of the animal. For a number of reasons, the Xenopus melanotrope cells represent an attractive cell model system. First, this homogeneous cells population of strictly regulated neuroendocrine secretory cells and their biosynthetic and secretory activity can be physiologically manipulated *in vivo* by simply placing the amphibian on a black or white background. Second, on a black background these cells produce large amounts of a single cargo molecule, the prohormone proopiomelanocortin (POMC), that is transported via the regulated secretory pathway and processed to a number of bioactive peptides including α-melanophore stimulating hormone (α-MSH), which is responsible for darkening of the skin (Jenks et al., 1977). The *Xenopus* melanotrope cells also produce Aph-1, but its mRNA expression is not altered in black- compared to white-adapted animals. Third, all γ-secretase components as well as many γ-secretase substrates are highly conserved between *Xenopus* and mammals, making *Xenopus* a good animal model to study the function of Aph-1. In contrast to the two genes existing in mammals (Aph-1a and -1b), *Xenopus* has only one ancestral *Aph-1* gene (i.e. a single ancestral gene that is duplicated due to a duplication of the whole genome in the genus *Xenopus laevis* that occurred some 50 million years ago, Evans et al., 2004). Since at the time of the generation of the frogs transgenic for Aph1aS-GFP, the full-length *Xenopus laevis* Aph-1 coding sequence was not yet known, we decided to use for our studies the highly similar *Rattus norvegicus* the Aph-1aS sequence fused to the amino-terminus of GFP. Substituting a mammalian component of γ-secretase by its *Drosophila* counterpart has been shown to be successful in reconstituting active γ-secretase, emphasizing the evolutionary conservation of γ-secretase assembly and function (Takasugi et al., 2003). The fact that we were successful in generating Aph1aS-GFP-transgenic frogs indicates that overexpression of the fusion protein in the intermediate pituitary is not lethal. The transgene expression was under the control of a POMC gene promoter fragment to generate frogs overexpressing the fusion protein specifically in the intermediate pituitary melanotrope cells.

The transfection experiments with COS1 and Neuro2A cells revealed an intracellular distribution of the Aph1aS-GFP protein in membrane structures, predominantly close to the nucleus and resembling most likely the endoplasmic reticulum (ER) or the Golgi apparatus. These observations are in line with previous reports on transfected 293T and HEK293 cells overexpressing HA-tagged Aph-1b and -1aL, respectively, showing that the protein localises to the ER and *cis*-Golgi (Gu et al., 2003; Hansson et al., 2005). The size observed for the major Aph1aS-GFP fusion product in the western blot analysis (~45 kDa, of which ~27 kDa is accounted for by the GFP tail and ~18 kDa by Aph-1aS; resembles that found by others (Gu et al., 2003; Hebert et al., 2004; Ormo et al., 1996). The additional product of roughly twice the size of the monomeric protein has been reported previously to represent aggregated forms of Aph-1 due to overexpression of the multi-pass transmembrane protein (Hansson et al., 2005; Shirotani et al., 2004).

The temporal and spatial expression pattern in *Xenopus* transgenic for Aph1aS-GFP observed during early embryonic development (from stage 25 onwards and gradually specific to the intermediate pituitary) resembles that found in *Xenopus* transgenic for POMC-promoter-driven expression of GFP itself (Jansen et al., 2002). In addition, the pattern is in line with the expression pattern of the endogenous POMC-derived α-MSH-peptide in developing *Xenopus* (Kramer et al. 2003). While our co-IP analyses revealed that in the transgenic melanotrope cells the Aph1aS-GFP fusion protein was capable of forming a complex with endogenous PS1-CTF, no direct effects were observed on the steady-state levels of PS1-CTF, nor for the major cargo protein POMC, or other secretory pathway components, such as the POMC cleavage enzyme PC2 and the $p24\delta_{1/2}$ proteins. Similarly, studies in HEK293 and Chinese hamster ovary cells have shown (endogenous) multimerisation of Aph-1aL with PS1, and no increase of PS1 levels upon overexpression of Aph-1aL (Gu et al., 2003; Luo et al., 2003). At the functional level, the γ-secretase cleavage activity towards APP appeared not to be affected in the transgenic compared to the wild-type melanotrope cells. It should here be noted that, to date, γ-secretase is known to cleave a long list of over 15 substrates (Coolen et al., 2006). Therefore, it may very well be that the cleavage activity towards other substrates is affected in the transgenic melanotrope cells. Since in the pulse-chase metabolic cell labelling studies on the *Xenopus* intermediate pituitary no large differences in the amounts of 37-kDa POMC and the 18-kDa POMC-derived product in the cells and incubation media were observed for the wild-type and transgenic melanotrope cells, the introduction of the Aph1aS-GFP fusion protein did not greatly affect prohormone biosynthesis and processing, and the secretion of the prohormone-derived proteins.

We found that fully black-adapted transgenic animals showed a significantly more rapid adaptation to a white background compared to wild-type animals, which could indicate that the melanotrope cells are more prone to inhibition and thus give a more rapid reduction of α-MSH levels in the blood than the wild-type cells. Future superfusion experiments with NILs of animals adapted to a white background for a relatively short time may shed light on this matter.

Inhibition of α-MSH secretion by the NIL can be regulated at multiple levels (Roubos et al., 2005). Our apomorphine pulse-chase experiment indicated that the transgenic melanotrope cells are more sensitive to apomorphine and more efficiently block secretion compared to wild-type cells. Superfusion experiments with dopamine or apomorphine as secretagogues could be informative and support these preliminary data. Also, other known inhibitors of melanotrope secretion, such as neuropepide Y (NPY) and γ-aminobutyric acid (GABA) (de Rijk et al., 1990; de Rijk et al., 1992) could be tested in such an assay to examine whether the overexpression of the Aph1aS-GFP fusion protein only affects dopamine sensitivity or also influences other neurotransmitter systems.

Thus far, only one *Xenopus* F_1 line transgenic for Aph-1 has been analysed. It will be essential to study multiple lines to draw more definite conclusions from these experiments. In any case, the increased sensitivity to apomorphine is not a general effect in transgenesis; for example overexpression of the V-ATPase accessory subunit Ac45 fused to the animo-terminus of GFP in the NIL results in a reduction of the effect of apomorphine compared

to wild-type animals (Eric Jansen, unpublished results). Interestingly, we have previously shown in the APO-SUS/-UNSUS rat model a link between the mammalian Aph-1 family member Aph-1b and the dopamine receptor agonist apomorphine. Rats with reduced Aph-1b levels appeared to be more sensitive to the drug, as measured by an increased gnawing response (Coolen et al., 2005). Surprisingly, we now find that increased Aph-1a expression causes also a more sensitive dopamine system. At present, it is not clear whether this increase in sensitivity is due to a displacement of the endogenous *Xenopus* Aph-1 protein, causing reduced levels of this protein, by the high levels of the Aph-1a transgene product. Alternatively, the GFP tag of the Aph-1aS-GFP fusion protein may have had a dominant-negative effect on the functioning of the Aph-1aS/γ-secretase complex. The generation and analysis of transgenic *Xenopus* overexpressing Aph-1aS without a tag may clarify whether the latter explanation is correct.

Altogether, we have successfully targeted Aph1aS-GFP to the *Xenopus* melanotrope cells of the intermediate pituitary. Our preliminary results indicated a possible novel link between the Aph-1-containing γ-secretase complex and the dopaminergic neurotransmitter system. Further analyses will be necessary to shed more light on this remarkable observation.

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Chapter 7

GENERAL DISCUSSION

Neurodevelopmental disorders, such as schizophrenia, autism and attention-deficit hyperactivity disorder (ADHD), are characterized by complex phenotypes with a variety of symptoms that are not just restricted to neural tissues, and multiple genes are thought to be responsible for each of these disorders. The aim of the research described in this thesis was to gain insight into the molecular cause of complex neurodevelopmental phenotypes, using the APO-SUS and -UNSUS rats as an experimental model. The APO-SUS and -UNSUS rat lines have been pharmacogenetically selected from an outbred Wistar population. Extensive phenotyping revealed a complex phenotype for the APO-SUS rats with a range of characteristics, both neuronal and non-neuronal, and remarkably similar to that observed in schizophrenic patients (Chapter 1).

The molecular basis of the APO-SUS/-UNSUS rat model: genomic rearrangement in the *Aph-1b* locus

We performed expression microarray analyses of the APO-SUS/-UNSUS rat model and found as the only reproducible difference a reduced level of *Aph-1b* mRNA in APO-SUS compared to -UNSUS rats (Chapters 2 and 3). Detailed genomic analyses revealed that a genomic rearrangement between segmental duplications had occurred in the *Aph-1b* locus of these rats, resulting in one (I) or two (II) in-tandem copies of the *Aph-1b* gene in APO-SUS rats and three (III) copies in -UNSUS rats. Segmental duplications are quite common in the rodent and human genomes; ~2.92% of the rat genome, ~1.2% of the mouse genome and ~5% of the human genome consists of such direct-repeat elements (≥1kb in size and ≥90% sequence identity), all widely distributed across the chromosomes (Tuzun et al., 2004). The duplication of a gene and subsequent mutation of the descendent copy is widely regarded as a mechanism by which genes with new functions are created. In addition, segmental duplications are hot spots for non-allelic homologous recombination and unequal crossing over, leading to genomic deletions, duplications, inversions and translocations. Many disorders (generally referred to as genomic disorders) show an involvement of segmental duplications, such as the Williams-Beuren syndrome and the Charcot-Marie-Tooth syndrome (Samonte and Eichler, 2002).

Recently, the genome of the Brown Norway laboratory rat strain has been sequenced (Gibbs et al., 2004) and a search through the database shows that the *Aph-1b* locus contains two *Aph-1b* gene copies; *Aph-1b* and *-1b'*. Surprisingly, Southern blotting and PCR analyses revealed that various rat strains (including the Wistar, Lewis, Fischer as well as the Brown Norway strain) have three *Aph-1b* gene copies (*i.e.* also harbouring the chimaeric *Aph-1b/b'* gene). Only the wild brown rat (sample collection through various pest control companies) displayed an *Aph-1b* locus similar to the genome database and the II/II rats (data not shown). This suggests that the ancestral wild rat harboured two *Aph-1b* genes that shifted to three genes when laboratory rat strains were generated. Perhaps, during the generation and maintenance of these standard laboratory rat strains, an unintentional selection has occurred toward more manageable (i.e. less stress susceptible) rats within the strains, in favour of the III/III genotype. The discrepancy in the *Aph-1b* gene copy

number of the Brown Norway rat sequence described in the rat genome database and found in our genotyping experiments may be caused by a heterogeneity in the Brown Norway strains, or - perhaps more likely - the segmental duplication in this region was problematic for sequencing and computer assembly of the rat genome (the mouse genome database sequence has also two *Aph-1b* gene copies). Furthermore, all Wistar rats tested contained the III/III genotype, implicating that a genomic rearrangement in the *Aph-1b* locus has occurred only in the APO-SUS rats, while this locus has remained unchanged in the APO-UNSUS rats. Thus, a difference in recombination susceptibility may exist between the two rat lines. Such a difference could have been caused by for example a differential epigenetic status of the *Aph-1b* locus. Epigenetic factors, such as nucleosome reorganisation, histone modifications and DNA methylation, are generally known to influence the phenotype without changing the genotype. In addition, a number of studies have demonstrated that histone/chromatin modifications not only influence DNA expression, but also DNA replication, recombination, and repair (Hassa and Hottiger, 2005; Khorasanizadeh, 2004). It would therefore be very interesting to analyse in these rats the epigenetic status of the *Aph-1b* locus in more detail. Also, the recombination event resulting in the APO-SUS rats could have been driven by external factors, such as the injection of the dopamine receptor agonist apomorphine that was used for the generation of the rat lines. Previous research has revealed the mutagenic capacity of apomorphine and especially of its oxidation derivative (Picada et al., 2005). Other external factors, like pre-, peri- or early post-natal stress or maternal care, may affect the methylation or recombination events as well (Weaver et al., 2004). It would therefore be interesting to identify the trigger leading to the recombination event in the rats (e.g. by the injection of Wistar rats with oxidized apomorphine or by applying severe stress and subsequent DNA [methylation] and chromatin analysis). In addition, examining direct repeat sequences in the APO-SUS genome would be of interest. Perhaps other regions also show a recombination event.

The observation that all Wistar rats are of the III/III genotype is not in line with the observed link between the *Aph-1b* copy number and apomorphine susceptibility described in Chapters 3 and 5. In an apomorphine susceptibility test, the Wistar rat population showed a bimodal distribution; some rats hardly responded whereas others gnawed vigorously (Cools et al., 1990; Ellenbroek and Cools, 2002, and personal observation). In addition, the *Aph-1b* mRNA levels did not differ significantly between these Wistar rats (data not shown). On the other hand, our backcross experiments (Chapter 5) showed that in a highly similar general genetic background, rats with one *Aph-1b* gene were significantly more susceptible to apomorphine than rats with three gene copies. Apparently, next to the *Aph-1b* copy number also other factors can influence apomorphine susceptibility. Nevertheless, our functional studies on the paralogue of Aph-1b, termed Aph-1a, by transgene expression in the neuroendocrine intermediate pituitary melanotrope cells of the South-African claw-toed frog *Xenopus laevis*, revealed in the transgenic cells an increased sensitivity to inhibition of regulated secretion by apomorphine (Chapter 6). Together with the differential apomorphine susceptibility of the APO-SUS and -UNSUS rat lines, this suggests that the γ-secretase complex is somehow involved in generating susceptibility to the dopamine receptor agonist.

Functional studies on *Xenopi* transgenic for Aph-1a

In Chapter 6, we employed stable *Xenopus laevis* transgenesis in combination with a cell-specific POMC gene promoter fragment as a tool to express Aph-1a carboxy-terminally tagged with the green fluorescent protein (Aph1a-GFP) specifically in the melanotrope cells. These cells produce large amounts of the prohormone POMC when the animal is placed on a black background, whereby a POMC-derived hormone causes the dispersion of skin melanophores. Conversely, on a white background these cells are inactive because of directly innervating inhibitory neurons of hypothalamic origin, including dopaminergic neurons. The analysis of our transgenic animals through pulse-chase metabolic cell labelling studies revealed that, despite the high levels of transgene expression, POMC synthesis and processing, and the secretion of POMC-derived products remained unaffected in the transgenic melanotrope cells. Surprisingly, we found that fully black-adapted transgenic animals showed a significantly more rapid adaptation to a white background compared to wild-type animals. Furthermore, the transgenic melanotrope cells appeared to be more sensitive to apomorphine, i.e. their regulated secretion of newly synthesised POMC-derived products was more inhibited than secretion from wild-type cells. These results suggest a modified sensitivity of the dopaminergic system in the transgenic *Xenopi*. However, it has to be stressed that the findings described in Chapter 6 are only preliminary. Thus far, only one *Xenopus* F_1 line transgenic for Aph-1a has been analysed and a limited number of experiments have been performed on these frogs. It will be essential to study multiple transgenic lines in more detail to draw more definitive conclusions. Such experiments will have to prove that the observed effects were not caused by mere line-specific differences or by the site of integration of the transgene DNA fragment into the genome of the frog (position effect). Furthermore, it has to be examined whether the effect is dopamine-specific or if other inhibitory mechanisms of melanotrope secretion (e.g. neuropeptide Y or GABA) are also affected in these animals.

Recombination of the rat *Aph-1b* locus

The data presented in Chapters 3 to 5 provide evidence that in APO-SUS rats the reduced number of *Aph-1b* genes is directly related to their phenotype. First, the APO-SUS/-UNSUS rat lines were generated twice, independently and with a ten-year interval. In both the original and the replicated APO-SUS and -UNSUS lines, the same *Aph-1b* gene copy number distribution was observed: one (I) or two (II) genes in APO-SUS rats and three (III) gene copies in APO-UNSUS rats. Second, the I/I and II/II rats from the APO-SUS line and the III/III rats from the APO-UNSUS line were tested separately for their behaviour (Chapter 3) revealing some features of a gene-dosage effect (e.g. locomotor activity in the open field or open arm entries in the elevated plus maze test), while for other behavioural characteristics a threshold in *Aph-1b* gene copy number appears to exist, i.e. a similar increase in gnawing score (apomorphine test) or in habituation time in the open field test is observed in the I/I and II/II rats compared to the III/III rats. And third, by crossing I/I rats of the APO-SUS line with III/III rats of the APO-UNSUS line, as performed in Chapter 5, we mixed the genetic background of both lines. By reselection

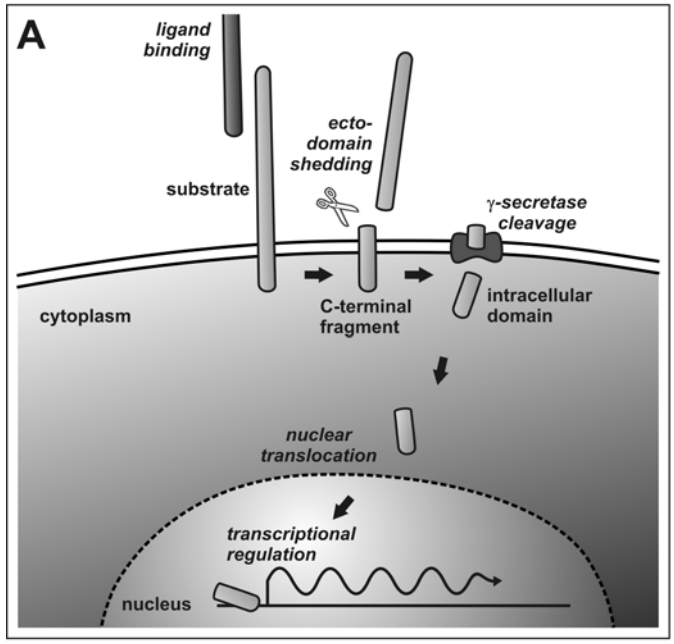
of the various *Aph-1b* genotypes, new lines were created with otherwise a highly similar general genetic backgrounds. We showed that the apomorphine susceptibility segregated with the *Aph-1b* genotypes, clearly indicating a correlation between genotype and phenotype. Correlations for other phenotypic characteristics are likely to be observed in future experiments. Similar to the I/I and III/III crossing experiments, the II/II rats of the APO-SUS line have recently been crossed with III/III rats of the APO-UNSUS line for future phenotyping (see also Chapter 4). A detailed gene-dosage dependency of various characteristics can now be determined using the I/I, II/II and III/III rats that only differ in *Aph-1b* copy numbers.

Aph-1b and the gamma-secretase complex

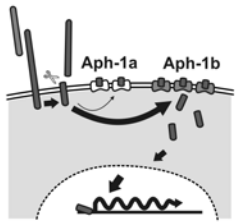
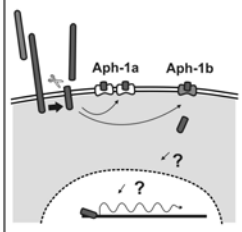
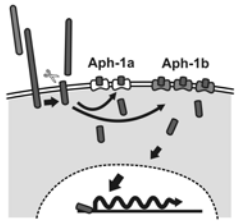
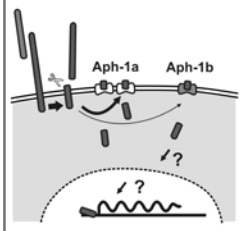
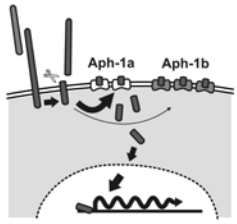
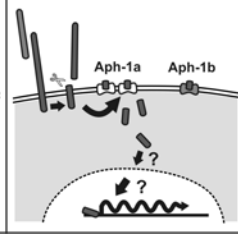
In mammals, together with the Aph-1aS and -1aL proteins, the Aph-1b protein is part of the family of anterior pharynx defective 1 (Aph-1) proteins. Recently, it has been shown that this family is a component of the minimal molecular subunit composition of the γ-secretase complex that includes presenilin (either PS-1 or PS-2), nicastrin (Nct), presenilin enhancer 2 (PEN-2) as well (De Strooper et al., 1998; Edbauer et al., 2003; Francis et al., 2002; Yu et al., 2000). The γ-secretase complex belongs to the family of aspartyl proteases and cleaves many type I transmembrane proteins within their membrane domain (Figure 1A). In general, the proteolytic processing of a γ-secretase substrate is initiated by the binding of the substrate to its ligand and subsequent shedding of its extracellular domain by a disintegrin metalloprotease, leaving a membrane-bound C-terminal fragment (CTF) that is subsequently cleaved by γ-secretase within its transmembrane domain. The resulting intracellular domain (ICD) is released into the cytoplasm and translocates to the nucleus, where it is involved in transcriptional regulation. In this thesis, we have shown that in APO-SUS rats the reduced *Aph-1b* mRNA levels caused a reduction of γ-secretase cleavage activity towards multiple substrates (Figure 1B). Thus far, thirty-three

Figure 1. Model of substrate cleavage by gamma-secretase

(A) Upon ligand binding, the ectodomain of the substrate is shed, resulting in the generation of a membrane-bound C-terminal fragment (CTF). This CTF is subsequently cleaved by the γ-secretase complex within its membrane region and the released intracellular domain (ICD) translocates to the nucleus, where it is involved in transcriptional regulation (after Kopan and Ilagan, 2004). (B) The reduction of the *Aph-1b* levels (in I/I compared to III/III rats) causes a decrease in Aph-1b-containing γ-secretase complexes, whereas the levels of Aph-1a-containing complexes remain unaffected (depicted by number of Aph-1a [white] and -1b [grey] particles). The various γ-secretase substrates can show differences in selectivity towards either Aph-1b or -1a containing complexes (left column). Substrates with a preference for Aph-1b will show the most severely affected γ-secretase processing in I/I rats (upper part of scheme), whereas substrates with a high affinity for Aph-1a-containing γ-secretase complexes may not be affected by the reduced *Aph-1b* levels (lower part of scheme). In this thesis, we have shown the direct consequences of the reduced *Aph-1b* levels in I/I rats for several substrates. We hypothesise the occurrence of downstream changes. For clarity, only two types of γ-secretase complexes (Aph-1a and -1b) are shown, whereas six complexes with different subunit compositions can be formed (containing one of the three Aph-1 proteins, and one of the two PS proteins). Differences in expression levels of the various components over time and place will add another layer of complexity to the situation in I/I rats (see Chapters 4 and 5 for examples).



B

preferential cleavage by:	III/III rats	I/I rats	consequences for I/I rats:
Aph-1b (▭)			**Severely affected:** CTF accumulation ICD reduction **Likely severely affected:** downstream pathway
no preference (▭/▭)			**Moderately affected:** CTF accumulation ICD reduction **Likely moderately affected:** downstream pathway
Aph-1a (▭)			**No consequences**

γ-secretase substrates have been discovered, each of which is part of a specific signalling pathway (Table 1).

The *Aph-1b* gene is present in all mammalian species, while the *Aph-1b* duplication (and thus the presence of the *Aph-1b'* gene) is rodent specific. Sequence comparisons suggest that *Aph-1a* is the closest orthologue to the ancestral *Aph-1* gene, which can be found in plants, worms and insects (Figure 2). The amino acid sequence alignment reveals a strong evolutionary conservation, suggesting an important basal function for Aph-1 proteins. The *Aph-1a* gene is transcribed as two splice variants, *Aph-1aS* and *-1aL*, and all transcript of the *Aph-1* family are ubiquitously expressed. The restricted presence of Aph-1b in mammals could imply a specialized function for this protein in the γ-secretase complex of higher organisms. In vertebrates as well as in plants and invertebrates, the presenilin family consists of two members, PS1 and PS2 (Ponting et al., 2002). This makes the Aph-1b protein the only mammalian-specific γ-secretase complex subunit. Mice with a *Aph-1a* knock-out have been shown to be lethal, whereas the deletion of both the *Aph-1b* and *-1b'* gene yielded viable animals with only localized signs of affected γ-secretase functioning (Ma et al., 2005; Serneels et al., 2005). This suggests that γ-secretase complexes containing Aph-1b have a different, perhaps more specialized, function than complexes containing Aph-1a. Future mutation analysis and domain swapping may reveal further functional differences between the Aph-1 family members.

Thus far, no mammalian-specific substrates of the γ-secretase complex are known, nor is any of the substrates exclusively processed by either Aph-1a- or -1b-containing γ-secretase complexes. However, the list of γ-secretase substrates is still growing, so a mammalian-specific (or Aph-1b-specific) substrate could still be discovered. The selective presence of Aph-1b in mammals may point towards specialisation of the functioning of the complex in these organisms that show complex behavioural traits.

Table 1. The gamma-secretase components and their substrates in a number of species

Species abbreviations: Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Xl; *Xenopus laevis*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*. Grey: specific orthologue present; White: single ancestral orthologue for family.

component	(*proposed*) function	orthologues						
PS1	catalytic subunit (De Strooper, 2003; Haass, 2004; Steiner, 2004)	Hs	Mm	Rn	Xl	Dm	Ce	At
PS2		Hs	Mm	Rn	Xl			
Nicastrin	substrate recognition (Shah et al., 2005)	Hs	Mm	Rn	Xl	Dm	Ce	At
PEN-2	endoproteolysis of PS holoprotein (Periz and Fortini, 2004)	Hs	Mm	Rn	Xl	Dm	Ce	At
Aph-1a	*complex stabilisation and maturation* (Lee et al., 2004; Niimura et al., 2005) and/or *substrate selectivity*? (Serneels et al., 2005, and this thesis)	Hs	Mm	Rn	Xl	Dm	Ce	At
Aph-1b		Hs	Mm	Rn				
Aph-1b'	*similar to Aph-1a/-1b?*		Mm	Rn				

(continued on next page)

(Table 1 continued)

substrate	(*proposed*) function	γ-secretase reference
APP	*transport or adhesion function?*	(De Strooper, 2003)
APLP1	*unknown function*	(Scheinfeld et al., 2002)
APLP2	*unknown function*	(Scheinfeld et al., 2002)
N-cadherin	cell adhesion	(Marambaud et al., 2003)
E-cadherin	cell adhesion	(Marambaud et al., 2002)
γ-protocadherin	cell adhesion	(Haas et al., 2005)
CD43	cell-cell interaction	(Andersson et al., 2005)
CD44	cell adhesion	(Lammich et al., 2002; Murakami et al., 2003)
CSF-1 receptor	protein tyrosine kinase	(Wilhelmsen and van der Geer, 2004)
DCC	netrin-1 receptor (axon guidance)	(Taniguchi et al., 2003)
ErbB4	growth-factor-dependent receptor tyrosine kinase	(Ni et al., 2003)
LRP	endocytic receptor	(May et al., 2002)
ApoER2	essential component Reelin/Disabled-mediated signal transduction during neuronal development	(May et al., 2003)
Megalin	endocytic receptor	(Zou et al., 2004)
Nectin-1α	adherance-junction formation	(Kim et al., 2002)
Neuregulin1,2	cell growth and differentiation	(Bao et al., 2003; Montero et al., 2002)
Notch1-4	cell fate / signalling receptor	(Schroeter et al., 1998)
Delta1	Notch ligand	(Six et al., 2003)
Jagged	Notch ligand	(Martoglio and Golde, 2003)
Syndecan-3	cell-surface proteoglycan co-receptor	(Schulz et al., 2003)
p75 NTR	neurotrophin co-receptor	(Kanning et al., 2003)
NRADD	death domain protein	(Gowrishankar et al., 2004)
VGSCβ1-4	cell-cell adhesion and migration	(Kim et al., 2005; Wong et al., 2005)
Alcadein α-γ	associates with APP through X11L / *unknown function*	(Araki et al., 2004)

Our detailed longitudinal analysis of *Aph-1* mRNA levels in developing I/I, II/II and III/III rats revealed temporal differences in these levels, without any compensation by *Aph-1a*. In addition, the ratios between the *Aph-1b* and *-1a* mRNAs differed significantly between tissues and over time. This resulted in spatio-temporally affected γ-secretase functioning toward various γ-secretase substrates in I/I compared to III/III rat tissues (Chapters 4 and 5). Moreover, we observed that in the I/I rats the levels of some, but not all, γ-secretase substrates tested were affected by the reduced *Aph-1b* mRNA levels. 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. Since γ-secretase substrates are part of diverse signalling pathways (from neuronal differentiation [notch and ErbB4] and synapse formation [nectin-1α] to haemotopoiesis and tumour growth [CD44]), cell-cell adhesion [E-cadherin], and vitamin homeostasis [LRP]), the reduced *Aph-1b* expression may well underlie the complex phenotype of the APO-SUS rats.

In Chapter 5, a reduction in p75-NTR processing by γ-secretase was found in certain I/I rat tissues. The p75 protein is known to be involved in a surprisingly diverse set of processes, ranging from cell death to regulation of axon elongation (Casaccia-Bonnefil et al., 1996). A more detailed analysis of such cellular processes in the I/I and III/III rats

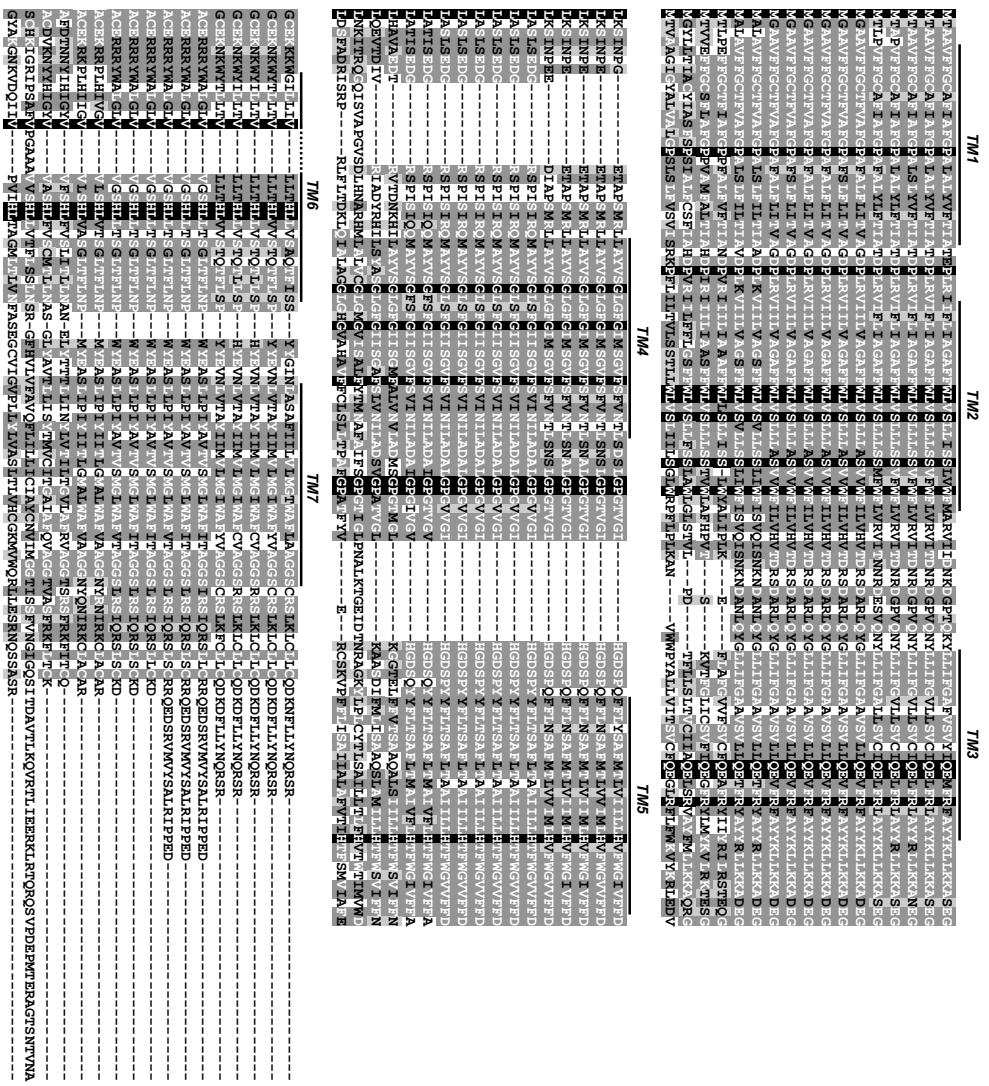


Figure 2. Amino acid sequence comparison of the Aph-1-related proteins in a number of species

Species abbreviations: Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*; Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*. Accession numbers: Hs_Aph1b (NP_112591); Rn_Aph1b (XP_578740); Mm_Aph1b (NP_808251); Rn_Aph1b' (XP_217185); Mm_Aph1b' (NP_080950); Hs_Aph1aL (AAH01230); Rn-Aph1aL (XP_345252); Mm_Aph1aL (AAH24111); Hs_Aph1aS (AAH08732); Rn_

may reveal further evidence for a link between an affected γ-secretase cleavage towards p75-NTR and the observed I/I phenotype. In addition, for various γ-secretase substrates the downstream targets of the intracellular domains (ICDs) are known (e.g. for notch and APP). These ICDs translocate to the nucleus where they are involved in transcriptional regulation. A more in-depth analysis of the mRNA levels of these downstream targets could illustrate that the reduced *Aph-1b* levels in the I/I rats do not only affect γ-secretase cleavage activity towards these substrates, but also influence their downstream signalling pathways (Figure 1). Also at the behavioural level, additional phenotypic effects may be observed in the rat model, which can be directly related to an affected γ-secretase cleavage activity. Aberrant APP processing is known to influence learning capabilities in mice (reviewed by Morgan, 2003). It would therefore be interesting to investigate the learning performances of the I/I and III/III rats more thoroughly in a Morris water maze test or via other learning paradigms.

Recently, the voltage-gated sodium channel beta 2 subunit (Scn2b) has been discovered as a new γ-secretase substrate (Kim et al., 2005; Wong et al., 2005). Scn2b is expressed only in central neurons (Jones et al., 1996) and although brain development in Scn2b-null mice was grossly normal the knock-out mice displayed increased susceptibility to epileptic seizures (Chen et al., 2002). Similarly, APO-SUS rats show a significantly increased incidence of bursts of bilateral synchronous spike wave discharges (SWDs) in their electro-encephalogram (EEG) which is associated with absence epilepsy (de Bruin et al., 2000). It is therefore well worth studying the Scn2b pathway in the rat lines. In addition, several groups have reported that the γ-secretase substrate neuregulin-1 (NRG1) is a candidate gene for schizophrenia (Li et al., 2006; Stefansson et al., 2002; Tosato et al., 2005). Furthermore, heterozygous *NRG1* knock-out mice are hyperactive in an open-field test and show an impaired prepulse inhibition, and these behavioural phenotypes are partially reversible with clozapine, an atypical antipsychotic drug used to treat schizophrenia (Stefansson et al., 2002). Also, mutations in the *NRG1* gene can cause impaired latent inhibition in mice (Rimer et al., 2005). Thus, analysis of the γ-secretase cleavage activity towards NRG1 in the I/I and III/III rats will be very informative and may reveal further similarities between the rat model and schizophrenic patients. Recently, the NRG1 receptor ErbB4 has also been directly linked to schizophrenia (Silberberg et al., 2006). A linkage disequilibrium block within the *ErbB4* gene revealed a strong association with schizophrenia. In addition, increased *ErbB4* transcript levels were found in the dorsolateral prefrontal cortex of patients compared to controls. Furthermore, *ErbB4* heterozygous knock-out mice show a behavioural phenotype that overlaps with mouse models for schizophrenia (Stefansson et al., 2002). Thus, strong arguments exist for an involvement of the γ-secretase-mediated NRG1-ErbB4 pathway in the pathophysiology of schizophrenia.

Aph1aS (NP_001014277); Mm_Aph1aS (NP_666216); Xl_Aph1 (AAH89044); Xt_Aph1 (AAH90132); Dm_Aph1 (NP_608710); Ag_Aph1 (XP_318923); Ce_Aph1 (NP_492469); At_Aph1 (NP_565724). A black background indicates identical and a grey background indicates conserved amino acid residues; horizontal bars are indicative of the seven putative transmembrane regions (TMs) in human and rodent sequences.

In Chapter 5, we described that γ-secretase cleavage activity towards multiple substrates is severely affected in the olfactory bulb. It will be of interest to analyse the functioning of the olfactory system in the different rat lines, by studying synaptic transmission or via behavioural experiments testing odour preference or performance. As an example of an odour preference test, the Y-maze could be used in which female rats of either line are examined for their preference to odours of males vs. oestrous females (Bakker et al., 2002). Furthermore, the cookie test could be used as an olfactory performance test (Dawson et al., 2005). An observed olfactory deficit would prove further similarity between the rat model and schizophrenia, where deficits in odour identification, detection threshold sensitivity, discrimination and memory have been reported (Brewer et al., 1996; Hurwitz et al., 1988; Malaspina et al., 1994; Moberg et al., 1997). Furthermore, this would further support our hypothesis that the γ-secretase complex is involved in the schizophrenic phenotype.

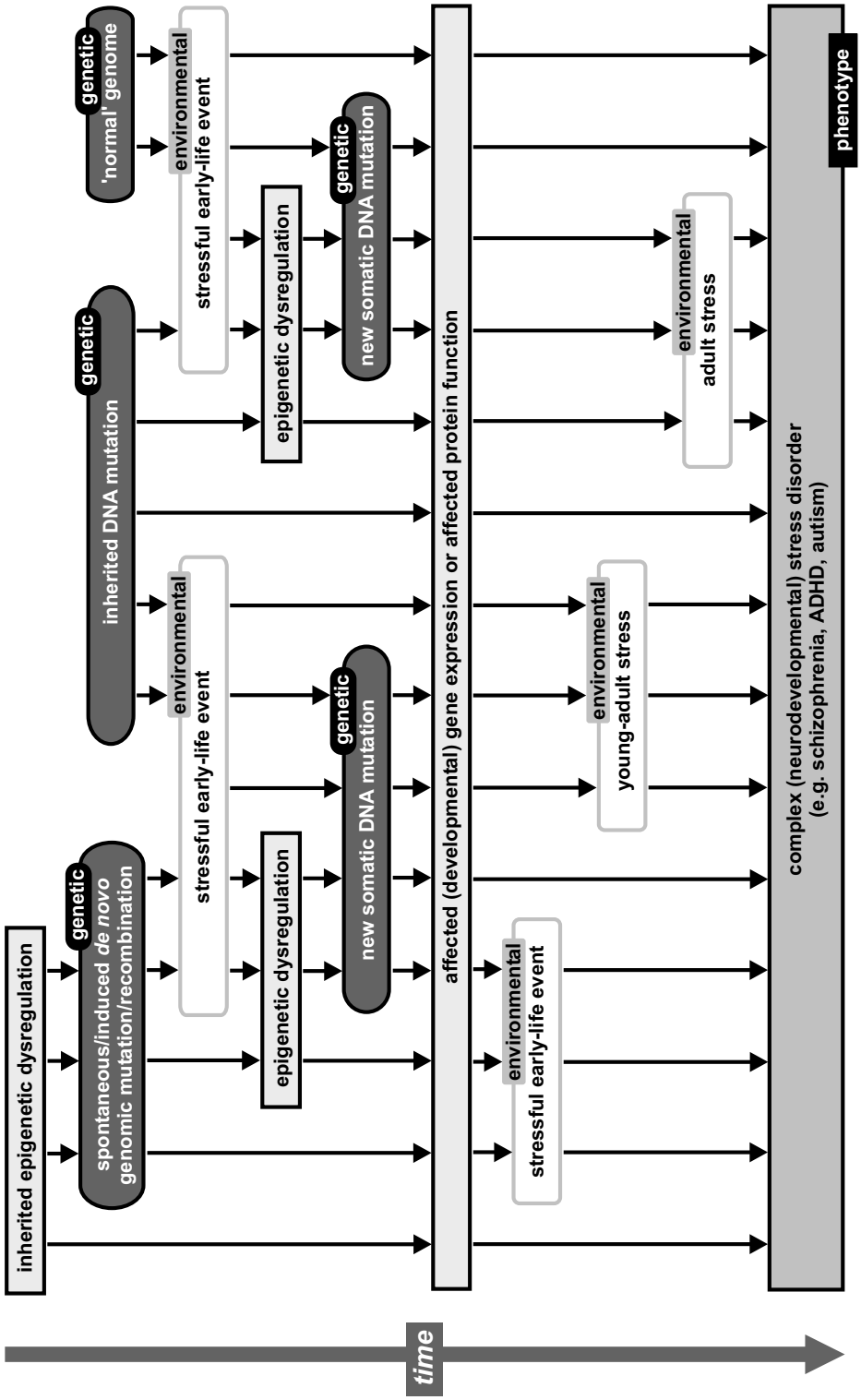
Interestingly, during early postnatal development of the APO-SUS/UNSUS rats different environmental stressors, such as maternal deprivation or cross fostering, have various effects on the adult phenotype (Degen et al., 2004; Ellenbroek et al., 2000). It would therefore be interesting to further analyse the possible effects of these environmental factors on *Aph-1b* mRNA levels, γ-secretase activity or certain γ-secretase substrate levels. By not only analysing the end situation in the adult rats, but especially during and shortly after the stress application, a more detailed understanding of the mechanisms underlying the origin of the complex adult phenotype may be obtained.

Extrapolation to schizophrenia in humans

At present, no evidence for an affected γ-secretase functioning in schizophrenic patients is available. As stated before, opposed to rodents, in humans only one gene encodes the Aph-1b protein, so an unequal crossing-over event in this genetic locus cannot occur. Initial results of an analysis of the *Aph-1b* locus in schizophrenic patients did not reveal duplications or large deletions in this genomic region (data not shown). However, other factors could influence the expression levels of the *Aph-1b* gene in human. Polymorphisms, mutations, epigenetic factors, affected levels of transcription factors involved in *Aph-1b* mRNA synthesis, and increased Aph-1b protein degradation are just a few ways in which the Aph-1b levels could be affected in schizophrenics. Furthermore, it must be born

Figure 3. General model on how a complex phenotype can be generated

Especially proteins that are part of complex, developmentally important networks (e.g. the Aph-1b protein) are prime suspects to be the causal factor for a complex stress phenotype, as observed in disorders like schizophrenia or depression. The expression of these (neuro-)developmentally important genes can be affected in various ways. Genetic mutations and/or epigenetic dysregulations can be either inherited or induced during early development, resulting in an affected protein functioning or expression level at critical developmental time points. Alternatively, stressful early-life events can directly influence protein functioning or expression levels, without the involvement of (epi-)genetic factors. Once protein levels or functioning are affected, a direct effect on the phenotype may be observed, but often additional stressors will trigger the actual onset of the disorder. The timing and type of stressor is critical for the phenotypic outcome. For clarity, not all possible combinations of events are shown.


inherited epigenetic dysregulation
genetic
spontaneous/induced de novo genomic mutation/recombination
environmental
stressful early-life event
epigenetic dysregulation
genetic
new somatic DNA mutation
genetic
inherited DNA mutation
environmental
stressful early-life event
epigenetic dysregulation
genetic
new somatic DNA mutation
genetic
'normal' genome
environmental
stressful early-life event
environmental
young-adult stress
environmental
adult stress
affected (developmental) gene expression or affected protein function
complex (neurodevelopmental) stress disorder
(e.g. schizophrenia, ADHD, autism)
phenotype
time

in mind that the signalling pathway in which Aph-1b is involved can also be affected at numerous other entry points. Thus, variations in other genes that feed into these pathways could also lead to similar phenotypes. Together, our findings on the molecular basis of the complex phenotype of the APO-SUS/-UNSUS rat model and the phenotypic observations in the I/I, II/II and III/III rat lines suggest a general model for the origin of a complex phenotype that may be applicable to human (Figure 3). In this model, a subtle defect in a (neuro)developmentally important gene may affect diverse signalling pathways, ultimately resulting in a complex phenotype that is generally thought to be caused by multiple affected genes. The expression or function of such a gene can be affected in various ways and at various time points during development. In addition, this model speculates on the influence of environmental stressors on the (epi-)genome, further emphasising the interaction between the genome and environment in the aetiology of a complex disorder. The results presented in this thesis may shed new light on the origin of complex disorders, like schizophrenia, and encourage further research into the possible involvement of the γ-secretase complex in these devastating disorders.

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SUMMARY / SAMENVATTING

Summary

Schizophrenia is a complex neurodevelopmental disorder affecting about 1% of the world's population. It is characterised by abnormal mental functions and disturbed behaviour, mostly appearing after puberty as a diverse mixture of positive and negative symptoms, and cognitive impairment. The severity of the symptoms and the chronic pattern of schizophrenia often cause a high degree of disability. Although many researchers try to find the origin of this disease, little progress is being made, most likely because of the complexity and diversity of the symptoms combined with the fact that most symptoms of schizophrenia are usually noticed quite late in the disease process. In this respect, heuristic animal models could help greatly to further analyse the neurodevelopmental hypothesis of schizophrenia. An example of such a heuristic animal model is the APO-SUS/-UNSUS rat model. APO-SUS rats show a range of symptoms that are remarkably similar to those observed in schizophrenic patients. The goal of the research described in this thesis was to understand the molecular mechanism underlying the APO-SUS/-UNSUS complex phenotype, which might aid in finding the cause of schizophrenia in human.

Chapter one gives an introduction to the characteristics and aetiology of schizophrenia, the different approaches researchers use to elucidate its cause, and the rationale for using animal models, in particular the APO-SUS/-UNSUS rat model.

In chapter two, we describe the use of cDNA and oligonucleotide microarray expression profiling to establish differences at the mRNA level between APO-SUS and -UNSUS rats in their juvenile and adult state. Remarkably few transcript levels were found to be affected. Upon validation, the largest differences observed turned out to be caused by a dissection artefact, namely partial co-dissection of the choroid plexus with some of the hippocampal samples analysed. The only reproducible mRNA expression difference concerned the reduction of *Aph-1b* mRNA in APO-SUS rats; Aph-1b is a component of the γ-secretase enzyme complex that is involved in multiple (neuro)developmental signalling pathways. These differences were found in all tissues, in male and female rats, and in the original as well as in an independent replication of the APO-SUS and -UNSUS rat lines. This strongly suggested that the reduced *Aph-1b* mRNA expression may be causative of the observed phenotype. However, the cause of the reduced expression remained a matter of speculation.

Chapter three reports a detailed genomic analysis of the genetic locus of *Aph-1b* in APO-SUS and -UNSUS rats revealing differences in gene copy numbers. APO-SUS rats harbour only one or two gene copies of *Aph-1b*, whereas APO-UNSUS rats contain three gene copies. This gene-dosage imbalance was caused by an unequal crossing-over event and the site of recombination was identified as a direct repeat (a segmental duplication) within the *Aph-1b* locus in the rat genome. The expression levels of the other γ-secretase components were analysed, but no differences were found. However, γ-secretase cleavage activity towards several substrates was affected and, furthermore, the *Aph-1b* genotypes segregated with a number of behavioural phenotypes.

In chapter four, we describe - through crossing and genetic re-selection - the generation of new rat lines with one (I/I), two (II/II) or three (III/III) *Aph-1b* gene copies against otherwise similar general genetic backgrounds. Subsequently, a detailed longitudinal expression analysis of the mRNA levels of the *Aph-1* family members (*Aph-1b*, and its paralogues *Aph-1aS* and *-1aL*) was performed in developing I/I, II/II and III/III rats. These analyses revealed gene-dosage dependent differences in *Aph-1b*, but not *Aph-1a*, mRNA expression throughout pre- and post-natal development. We were able to assign relative activities to the various *Aph-1a* and *-1b* gene promoters. In addition, 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.

The tissue-specific changes in γ-secretase activity are further addressed in chapter five, where we first analysed the tissue distributions of the Aph-1 family members in the I/I, II/II and III/III rats. We observed that the ratios between the *Aph-1b* and *Aph-1a* mRNA levels greatly varied among III/III rat tissues. A subsequent analysis of the γ-secretase cleavage activity towards the three APP superfamily members, p75 neurotrophin receptor, ErbB4 and neuregulin-2 in various I/I and III/III rat tissues showed 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. We thus provided *in vivo* evidence that subtle alterations in γ-secretase subunit composition may lead to both tissue- and substrate-specific changes in γ-secretase activity, which are likely to contribute to the complexity of the observed phenotype.

In chapter six, we describe a functional analysis of the rat Aph-1a protein by using stable transgenesis of the amphibian *Xenopus laevis*. We generated frogs that expressed the fluorescently tagged Aph-1a protein specifically in the melanotrope cells of the intermediate pituitary, and analysed the transgenic animals at the protein, cellular, physiological and organismal levels. The melanotrope cells are involved in the adaptation of the frogs skin colour to its background through the production of the prohormone proopiomelanocortin (POMC), the precursor of the α-melanophore-stimulating hormone. We observed no effect of the fusion protein on the processing of POMC in the intermediate pituitary cells. Intriguingly, the transgenic melanotrope cells were more sensitive to the inhibition of regulated secretion by the dopamine receptor agonist apomorphine than wild-type cells. Furthermore, the transgenic frogs adapted more rapidly to a white background than wild-type animals. Together, the results of these preliminary studies suggest that manipulation of Aph-1 expression in *Xenopus* melanotrope cells leads to a modified sensitivity of the dopaminergic system. Such a link between Aph-1 and the *Xenopus* dopaminergic system is in line with the observed difference in apomorphine susceptibility of the APO-SUS and -UNSUS rats. Future experiments will have to be performed to substantiate this conclusion.

Finally, in chapter seven the results described in this thesis are discussed and put in a broader perspective. Based on our findings in the APO-SUS and -UNSUS rats, we present a model explaining how a complex phenotype may arise from a defect in a single developmentally important gene, such as *Aph-1b*. This model is not only applicable to the APO-SUS and -UNSUS rats, but may also help in the understanding of human complex

diseases, such as schizophrenia. Furthermore, recommendations are provided for future fundamental research on the I/I, II/II and III/III rat lines as well as for translational research on schizophrenic patient material.

In conclusion, the studies described in this thesis suggest that a subtle change in the expression of a (neuro)developmentally important protein may spatio-temporally affect diverse signalling pathways, ultimately resulting in a complex phenotype that is generally thought to be caused by multiple affected genes. Furthermore, we implicate the possible involvement of the γ-secretase enzyme in complex disorders, such as schizophrenia.

Samenvatting

Schizofrenie is een complexe neurobiologische aandoening die ongeveer 1% van de wereldbevolking treft. Karakteristieke kenmerken van schizofrenie zijn abnormale mentale functies en een verstoord gedrag, welke meestal tot uiting komen na de pubertijd als een combinatie van positieve en negatieve symptomen en cognitieve tekortkomingen. Zowel de ernst van de symptomen als het chronische patroon van schizofrenie zorgen vaak voor een grote handicap bij de patiënt. Vele onderzoekers pogen de oorzaak van deze ziekte te doorgronden, maar er wordt weinig vooruitgang geboekt. Dit wordt mede veroorzaakt door de complexiteit en diversiteit van de symptomen, samen met het feit dat de meeste symptomen pas tot uiting komen nadat de ziekte zich heeft ontwikkeld. Daarom kunnen zogenaamde heuristische diermodellen helpen bij het verder analyseren van de neurobiologische hypothese van schizofrenie. Een voorbeeld van een dergelijk heuristisch diermodel is het APO-SUS/-UNSUS rattenmodel. APO-SUS ratten vertonen een scala aan symptomen die opvallend overeenkomen met de symptomen van schizofrenie. Het doel van het onderzoek beschreven in dit proefschrift was het achterhalen van het moleculaire mechanisme dat ten grondslag ligt aan het complexe APO-SUS/-UNSUS fenotype. De gevonden resultaten kunnen bijdragen aan het doorgronden van de oorzaak van schizofrenie bij de mens.

Hoofdstuk 1 beschrijft de kenmerken van schizofrenie, wat tot nu toe bekend is over het ontstaan van deze ziekte, en op welke manieren naar de oorzaken ervan wordt gezocht, inclusief het gebruik van diermodellen en in het bijzonder van het APO-SUS/-UNSUS rattenmodel.

In hoofdstuk 2 beschrijven we het gebruik van cDNA en oligonucleotide microarrays om verschillen in mRNA niveaus te ontdekken in weefsels van APO-SUS en -UNSUS ratten, zowel in pups als in volwassen ratten. Opvallend weinig transcripten vertoonden verschillen tussen beide lijnen en na validatie bleken de grootste verschillen veroorzaakt te worden door een dissectie-artefact, namelijk een verontreiniging van het hippocampus extract met fragmenten van omringend epitheelweefsel, het choroid plexus. Het enige reproduceerbare verschil bleek een reductie van de hoeveelheid *Aph-1b* mRNA in de APO-SUS ratten te zijn. Aph-1b is een component van het γ-secretase enzym complex dat betrokken is bij vele (neuro-)ontwikkelingsbiologische signaaltransductie- routes.

De verschillen in mRNA werden gedetecteerd in alle weefsels, in mannelijke en vrouwelijke ratten, en zowel in de originele APO-SUS en -UNSUS rattenlijnen als in een onafhankelijke replicatie van deze lijnen. Dit impliceert dat het verlaagde *Aph-1b* mRNA niveau een causale rol speelt bij het ontstaan van het fenotype van de ratten.

Hoofdstuk 3 beschrijft een gedetailleerde analyse van het *Aph-1b* gen in de APO-SUS en -UNSUS ratten, waarbij een verschil in het aantal gencopieën werd gevonden. APO-SUS ratten dragen slechts één of twee *Aph-1b* genen in hun genoom, terwijl APO-UNSUS ratten drie copieën bezitten. Dit verschil in het aantal genen werd veroorzaakt door een ongelijke paring tussen chromosoom-armen en de plaats van de recombinatie bleek een directe sequentieherhaling in de *Aph-1b* locus van het rattengenoom. Analyse van de expressieniveaus van de andere γ-secretase componenten leverde geen verschillen op. Echter, verscheidene γ-secretase substraten bleken minder efficient door het γ-secretase complex gekliefd te worden. Bovendien segregeerden de verschillende *Aph-1b* genotypes met een aantal fenotypische gedragskenmerken.

In hoofdstuk 4 beschrijven we – via kruisingen en daaropvolgend genetische selectie – het maken van nieuwe rattenlijnen met vergelijkbare genetische achtergronden, behalve dat ze één (I/I), twee (II/II) of drie (III/III) *Aph-1b* gencopieën bevatten. Vervolgens werd een gedetailleerde longitudinale expressie-analyse uitgevoerd van de mRNA niveaus van de *Aph-1* familieleden (*Aph-1b*, en de paralogen *Aph-1aS* en *-1aL*) tijdens de ontwikkeling van de I/I, II/II en III/III ratten. Deze analyse liet zien dat tijdens de gehele pre- en postnatale ontwikkeling er gendosis-afhankelijke verschillen in het mRNA niveau van *Aph-1b*, maar niet van *Aph-1a*, aanwezig waren. Aan de promotoren van de *Aph-1a* en *-1b* genen konden relatieve activiteiten worden toegewezen. Daarnaast werden in de drie rattenlijnen zowel weefselspecifieke als tijdsafhankelijke veranderingen in de γ-secretase klievingsactiviteit gevonden voor één van de meest bekende substraten, het amyloid-β voorloper eiwit APP.

In hoofdstuk 5 wordt eveneens aandacht besteed aan de weefselspecificiteit van de verschillen in γ-secretase activiteit. Allereerst werd de mRNA weefseldistributie van de Aph-1 familieleden in de I/I, II/II en III/III ratten bepaald. In de weefsels van III/III ratten bleken de ratios tussen de *Aph-1b* en *Aph-1a* mRNA niveaus sterk te variëren. Daarna werd in verscheidene I/I en III/III rattenweefsels een analyse uitgevoerd van de γ-secretase klievingsactiviteiten voor de drie APP superfamilieleden, de p75 neurotrofine receptor, ErbB4 en neureguline-2. De klieving van slechts een deel van deze substraten was veranderd. Bovendien bleken de gevonden verschillen alleen aanwezig te zijn in weefsels die, ten opzichte van *Aph-1a*, normaal een relatief hoog *Aph-1b* niveau hebben. Hiermee hebben we *in vivo* bewijs geleverd dat subtiele verschillen in de samenstelling van het γ-secretase complex kunnen leiden tot zowel weefsel- als substraatspecifieke veranderingen in γ-secretase activiteit. Deze veranderingen dragen waarschijnlijk bij aan de vorming van het waargenomen complexe fenotype.

In hoofdstuk 6 beschrijven we de functionele analyse van het ratten Aph-1a eiwit via stabiele transgenese van de amfibie *Xenopus laevis*. Kikkers werden gegenereerd die het fluorescerend gemerkte Aph-1a eiwit specifiek aanmaakten in de melanotrope cellen van de hypofyse middenkwab, en deze transgene dieren werden geanalyseerd op het eiwit-,

cellulair, fysiologisch en organismaal niveau. De melanotrope cellen uit de hypofyse middenkwab van de kikker zijn betrokken bij de aanpassing van de huidskleur aan de omgeving, namelijk door de aanmaak van het prohormoon pro-opiomelanocortine (POMC), het voorloper-eiwit van het α-melanofoor-stimulerend hormoon. We vonden geen effect van de overexpressie van het fusie-eiwit op de klieving van POMC in de cellen van de hypofyse middenkwab. In vergelijking met wild-type cellen, bleken de transgene melanotrope cellen wel gevoeliger te zijn voor de inhibitie van de gereguleerde secretieroute middels de dopamine receptor agonist apomorfine. Daarnaast adapteerden de transgene kikkers sneller aan een witte achtergrond dan wild-type dieren. De resultaten van deze voorlopige studies suggereren dat de manupulatie van de Aph-1 expressie in de *Xenopus* melanotrope cellen leidt tot een veranderde gevoeligheid van het dopaminerge systeem. Een dergelijke link tussen Aph-1 en het *Xenopus* dopaminerge systeem is in overeenstemming met de gevonden verschillen in apomorfine gevoeligheid van de APO-SUS en -UNSUS ratten. Verdere experimenten zullen moeten worden uitgevoerd om deze conclusie te versterken.

Tot slot worden in hoofdstuk 7 de resultaten, die beschreven zijn in dit proefschrift, bediscussiëerd en in een breder perspectief geplaatst. Gebaseerd op onze bevindingen in de APO-SUS en -UNSUS ratten presenteren we een model dat verklaart hoe een complex fenotype kan ontstaan door een defect in één enkel gen dat belangrijk is voor de ontwikkeling van het organisme, zoals het *Aph-1b* gen. Dit model is niet alleen toepasbaar op de APO-SUS en -UNSUS ratten, maar het kan ook helpen bij het doorgronden van complexe humane ziekten, zoals schizofrenie. Verder worden er in dit hoofdstuk aanbevelingen gedaan voor vervolgonderzoek aan de I/I, II/II en III/III rattenlijnen, maar ook aan patiënten met schizofrenie.

Samenvattend suggereert het onderzoek beschreven in dit proefschrift, dat een subtiele verandering in de expressie van één eiwit dat (neuro-)ontwikkelingsbiologisch van belang is een scala aan signaaltransductieroutes kan beïnvloeden op een zowel weefselspecifieke als tijdsafhankelijke manier. Het uiteindelijke resultaat is een complex fenotype, waarvan in het algemeen wordt verondersteld dat het veroorzaakt zou worden door de aantasting van meerdere genen. Daarnaast geven de resultaten aan dat het γ-secretase enzym mogelijk betrokken is bij complexe ziekten, zoals schizofrenie.

CURRICULUM VITAE / PUBLICATIONS

Curriculum vitae (English)

Marcel Wilhelmus Coolen was born on April 26, 1975 in Nederweert, the Netherlands, at 11:00 AM exactly. After his VWO (pre-university education) graduation at the 'Philips Van Horne Scholengemeenschap' in Weert in 1993, he started his studies Bioprocess technology at Wageningen University, the Netherlands. During this period he completed three internships; a 9 months virological project at the department of Virology at Wageningen University (under the supervision of Dr. Richard Kormelink), a 7 months biochemical project at the Rudolph Magnus Institute for Neurosciences at Utrecht University (supervised by Dr. Marten P. Smidt), and a 5 months immunological project at the department HIV R&D of the Australian Red Cross Blood Bank in Sydney, Australia (supervised by Dr. Wayne B. Dyer). On August 31, 1998, he obtained the degree Master of Science (with distinction), and in October of the same year, he started his Doctor of Philosophy (PhD) studies at the department of Molecular Animal Physiology at the Radboud University of Nijmegen, under the supervision of Prof. Dr. Gerard J.M. Martens. During this time he collaborated with the department of Psychoneuropharmacology (Prof. Dr. Alexander R. Cools and Dr. Bart A. Ellenbroek) and the pharmaceutical company Organon. At the end of a challenging, but risky PhD period, he was offered a postdoctoral fellowship at the same department to allow a continuation and completion of some very interesting leads. He gratefully accepted this offer and the end results are not only described in this thesis, but also in a number of high-impact publications. As of October 2005, Marcel Coolen is continuing his scientific carrier as a senior research officer in the lab of A/Prof. Dr. Sue Clark at the Cancer-Epigenetics group of the Garvan Institute of Medical Research in Sydney, Australia, where he is exploring the epigenetic differences between identical twins.

Curriculum vitae (Nederlands)

Marcel Wilhelmus Coolen werd geboren op 26 april 1975 te Nederweert, om 11:00 uur precies. Na het behalen van zijn eindexamen VWO aan de Philips Van Horne Scholengemeenschap te Weert begon hij in 1993 met de studie Bioprocestechnologie aan de toenmalige Landbouw Universiteit Wageningen. Gedurende deze studie werden twee afstudeerprojecten uitgevoerd; een virologisch project van 9 maanden bij de vakgroep Virologie aan de Landbouw Universiteit Wageningen (onder leiding van Dr. Richard Kormelink), en een biochemisch project van 7 maanden aan het Rudolph Magnus Instituut voor Neurowetenschappen, Rijksuniversiteit Utrecht (onder leiding van Dr. Marten P. Smidt). Daarnaast liep hij een immunologische stage van 5 maanden bij de afdeling 'HIV R&D' van de Australian Red Cross Blood Bank te Sydney, Australië (onder leiding van Dr. Wayne B. Dyer). Op 31 augustus 1998 behaalde Marcel Coolen - met lof - de ingenieurs titel en in oktober 1998 begon hij als assistent in opleiding (AiO) bij de vakgroep Moleculaire Dierfysiologie aan de toenmalige Katholieke Universiteit Nijmegen, onder leiding van Prof. Dr. Gerard J.M. Martens. Binnen zijn onderzoek werd nauw samengewerkt met de vakgroep Psychoneurofarmacologie (Prof. Dr. Alexander R. Cools en Dr. Bart A. Ellenbroek) en het farmaceutisch bedrijf Organon. Na afloop van een uitdagende, doch risicovolle AiO-periode, werd hem in april 2003 een positie als post-doc aangeboden bij dezelfde vakgroep om de zeer hoopvolle onderzoekslijnen te vervolgen en af te ronden. Deze mogelijkheid werd met beide handen aangegrepen en het eindresultaat is niet alleen beschreven in dit de proefschrift, maar ook in een aantal toonaangevende publicaties. Per oktober 2005 heeft Marcel Coolen zijn wetenschappelijke carrière voortgezet als post-doc bij Dr. Sue Clark op de afdeling 'Cancer - Epigenetics' van het Garvan Institute of Medical Research te Sydney, Australië, waar hij de epigenetische verschillen tussen identieke tweelingen bestudeert.

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