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Linking genetic variation and epigenetic modification: Functional promoter analysis of *SCL12A6* and *NR3C1*, two candidate genes involved in the pathogenesis of mood disorders

Dissertation

von

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Table of Contents

Index of Figures and Tables

Figures:

Tables:

Software:

Bimas signal scan	http://www-bimas.cit.nih.gov/molbio/signal/
Bimas promoter scan	http://www-bimas.cit.nih.gov/molbio/proscan/
CpG island strength predicton	http://neighborhood.bioinf.mpi-inf.mpg.de/CpG_islands_revisited/
ClustalW	http://www.ebi.ac.uk/clustalw/
Ensembl database	http://www.ensembl.org/index.html
NEBcutter	http://tools.neb.com/NEBcutter2/index.php
Primer3	http://frodo.wi.mit.edu/
PUBmed	http://www.ncbi.nlm.nih.gov/pubmed/
UCSC genome browser	http://genome.ucsc.edu/

Abbreviations

A	adenine
Aa	amino acid
ACCPN	agenesis of the corpus callosum associated with peripheral neuropathy
ACTH	adrenocorticotropic hormone
ADHD	attention deficit/hyperactivity disorder
ANOVA	analysis of variance
AP	activator protein
cAMP	cyclic adenosine monophosphate
ASD	autism spectrum disorder
BDNF	brain derived neurotrophic factor
Bp	basepair
BPD	bipolar disorder
C	cytosine
CBG	corticosteroid binding globuline
CDCV	Common Disease Common Variants
cDNA	complementary DNA
CDRV	Common Disease Rare Variants
CFS	chronic fatigue syndrome
CFTR	cystic fibrosis transmembrane conductance regulator
CNV	copy number variation
CNP	copy number polymorphisms
COMT	catechol O methyltransferase
CREB	cAMP response element binding-protein
CRH	corticotropin releasing hormone
DAT	dopamin transporter
Del	deletion
Dex	dexamethasone
DISC1	disrupted in schizophrenia 1
DNA	desoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	desoxyribonuceicacid triphosphate
DAOA	D-amino acid oxidase activator
DRD	dopamin receptor D

DZ	dizygotic
EGR	early growth response protein
EMEM	Eagle's minimal essential medium
For	forskolin
G	guanine
GABA	gamma-aminobutyric acid
GC	glucocorticoid
GR	glucocorticoid receptor
GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate
GWAS	genome wide association studies
HAT	histone acetyltransferase
HDAC	histone deacetylases
HMT	histone methyltransferase
HPA	hypothalamus-pituitary-adrenal
HT2RA	hydroxytryptamin receptor 2 A
IGE	idiopathic generalized epilepsy
Ins	insertion
IGF2	insulin-like growth factor 2
Kb	kilobase
KCC	potassium chloride cotransporter
LD	linkage disequilibrium
Li	lithium
LOD	logarithm of the odds
M	mol
MAOA	monoamine oxidase A
Mb	mega base
MBD	methyl CpG binding domain protein
MeCP	methylcytosine binding protein
MD	major depression
MDD	major depressive disorder
MLC	megalencephalic leukoencephalopathy with subcortical cysts
mRNA	messenger RNA
MSH	melanocyte-stimulating hormone
MSP	methylation specific PCR

MZ	monozygotic
NEAA	non essential amino acids
NGFI-A	nerve growth factor inducible protein A
nNOS	neuronal nitric oxide synthase
NR3C1	nuclear receptor subfamily 3, group C, member 1
nsSNPs	non-synonymous single nucleotide polymorphisms
nt	nucleotide
OMIM	online mendelian inheritance in man
PCR	polymerase chain reaction
Pen	penicillin
PKA	protein kinase A
PKC	protein kinase C
PLB	passive lysis buffer
PMA	phorbol 12-myristate 13-acetate
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RELN	reelin
RFLP	restriction fragment length polymorphism
RLU	relative light units
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SCZ	schizophrenia
SDAT	senile dementia-alzheimer’s type
SK-N-SH	human Caucasian neuroblastoma cell-line
SLC12A6	solute carrier family 12, member 6
SNP	single nucleotide polymorphism
Strep	streptomycin
T	thymine
TPH2	trypthophan hydroxylase 2
TSA	trichostatin A
TSST	Trier social stress test
U	units
UCSC	University of California Santa Cruz
UTR	untranslated region

USF	upstream transcription factor
U373MG	human Caucasian glioblastoma-astrocytoma cell-line
Val	valproic acid
YY 1	ying yang-1
5-HT	serotonin
5-HTT/SLC6A4	serotonin transporter
5-aza	5- azacytidin

Auf die Dauer der Zeit nimmt die Seele die Farben der Gedanken an

(Marc Aurel; 121 – 180 n. Chr.)

Chapter 1

1.1 Introduction and outline

Psychiatric and mental disorders are diseases of the human brain with an underlying complex etiology. It is estimated that across 28 European countries with a total population of 466 million, 127 million people or 27% of the adult European population, 18-65 of age, are or have been affected by at least one mental disorder in the past 12 months (Wittchen and Jacobi 2005). The lifetime risk to develop at least one brain disorder is considered to be 50% for individuals in the EU population (Wittchen and Jacobi 2005). Mental disorders such as bipolar disorder (BPD), major depressive Disorder (MDD) and schizophrenia (SCZ) can start as early as in childhood and adolescence. They typically have adverse effects on school and professional career, social functioning and somatic health throughout life, and are associated with great suffering also for the families, partners and friends. As a result of such a high prevalence of mental disorders combined with the early onset and their life long course, the total burden for society associated with mental disorders is immense. In 2004, the total cost for psychiatric disorders was estimated to be around 290 billion Euros in the EU (Wittchen and Jacobi 2005; Wittchen et al. 2005).

Mental health problems, such as bipolar disorder or major depression are complex in their etiology as biological, social and psychological factors interact in the course of their development. Family, twin and adoption studies have established that BPD, MDD and other forms of severe affective illness are highly heritable. Twin studies are showing markedly increased concordance rate of BPD by comparing monozygotic with dizygotic twins (Bertelsen et al. 1977; Cardno et al. 1999). Furthermore, the influence of genetic factors is supported as biological parents are more often affected than the adoptive parents of BPD adoptees (Mendlewicz and Rainer 1977). A multitude of linkage and association studies have been conducted during the past decade (DeLisi et al. 2000; Detera-Wadleigh et al. 1999;

Detera-Wadleigh et al. 1997; Dick et al. 2002; Liu et al. 2003; Stober et al. 2000a; Stober et al. 2001; Stober et al. 2000b; Stober et al. 2002) and even more susceptibility genes for psychiatric disorders have been identified (Table 1.1). An extensive combination of linkage- and association studies and other approaches have been performed to clarify the genetic mechanism of complex mental disorders. To cope with the high complexity of psychiatric disorders, the investigation design has also to consider gene x gene and gene x environment interactions.

Psychosocial stress has been identified as an important modifier of mental and behavioral disorders. The relationship of mental disorders to stressful life events is well established, both in epidemiological and clinical samples (Paykel 2003), and virtually all psychiatric disorders are closely linked with stress (Young 2004). As proposed by the stress diathesis model (Zubin and Spring 1977) it can be assumed that mental disorders develop when genetic vulnerability or predisposition interacts with environment and life events. It is a well accepted theory that behavior and also mood disorders are not caused by single genes or alterations in single genes, but rather are the result of more subtle alterations in multiple genes, each contributing partially to the expression of behavior. Furthermore, it can be assumed that classical genetics alone is not sufficient to explain behavioral outcomes but that the environment makes a significant contribution to the ultimate phenotype. For instance, the vulnerable phenotype model proposes that responses to stressors depends on the individual's genetic predisposition and is modulated by the individual's history, in particular prenatally and during early life (de Kloet et al. 2005). Numerous studies have been conducted, providing evidence that mutations and/or polymorphisms are associated with psychiatric disorders (Heils et al. 1996; Lesch et al. 1996; Meyer et al. 2001; Meyer et al. 2005; Stober et al. 1996). Furthermore, it was demonstrated that after experienced childhood adversity, the individual's outcome is dependent on the respective genotype (Caspi et al. 2002; Caspi et al. 2003; Kendler et al. 2005). Other studies provided evidence that serotonin transporter (*SLC6A4)* promoter

genotype moderates amygdala activation in response to threatening environmental stimuli, like angry or fearful faces (Hariri et al. 2003; Hariri et al. 2002a; Hariri et al. 2002b; Hariri and Weinberger 2003a; Hariri and Weinberger 2003b). However, the question remains, why some people who are exposed to similar environmental pathogens, such as psychological stress, develop mental disorders while others do not. To answer this question, it is becoming increasingly important to unravel "predisease pathways" (Singer 2001) and to identify psychobiological mechanisms linking environmental influences to changes at the molecular level that predispose to, or sustain, disease processes. As we become better versed in identifying environmental factors which affect phenotypes, it can be expected that not only new genes will be identified, but also new polymorphisms and complex mechanisms of gene regulation and gene x gene and gene x environment interactions.

In this context this work was inspired by two different investigations. The first (Meyer et al. 2005), identifying associations of *SLC12A6* polymorphisms with BPD, was based on the results of a genome wide linkage analysis (Stober et al. 2001), investigating 12 multiplex pedigrees with 135 individuals (78 healthy and 57 subjects with catatonic symptoms; MIM 181500; Leonhard 1999; APA 1994). Periodic catatonia is characterized by a fluctuating phenotype, combining akinetic negativism, immobility and mutism, or hyperkinesia with stereotypies and parakinetic movements as well as increased anxiety, impulsivity and aggressiveness. According to Tylor et al. (2003), periodic catatonia can be conceptualized as a subtype of schizophrenia (Leonhard 1999), however, it also is observed in other psychiatric disorders, especially in BPD. Stober et al. (2001) found evidence for linkage with markers to chromosome 15q15 and a suggestive evidence for linkage to chromosome 22q13. In further studies, linkage of polymorphic markers between D15S144 and D15S1028 with periodic catatonia could be confirmed (Meyer et al. 2002; Stober et al. 2002). Consequently, performing candidate gene approaches, two rare G/A polymorphisms (32418760 G/A and 32416574 G/A; numbering of nucleotide positions are relative to chromosome 15 of the

UCSC hg18 assembly of the human genome, March 2006; no rs numbers available), located in the 5' regulatory region of *SLC12A6* were identified, which showed association with bipolar disorder in a case control study (Meyer et al. 2005). Interestingly, recessive mutations in the coding region of this gene, leading to an unfunctional SLC12A6 are causative for the Andermann syndrome (ACCPN; OMIM 218000) in humans. This syndrome is characterized by frequent congenital *corpus callosum* agenesis, mental retardation, psychotic episodes, peripheral neuropathy, and some dysmorphic features bearing analogy with catatonia symptoms (Filteau et al. 1991). *SLC12A6* being an interesting candidate gene additionally was supported by the variants' location in the 5' regulatory region of the gene. Promoter activity can be modulated by epigenetic modifications such as DNA methylation, possibly as an adaptation of the organism to the environment. Interestingly, a single nucleotide polymorphism (SNP) in the *SLC12A6* promoter region creates a methylation site representing a target for epigenetic modification in the G-specific allele which is not present in the *SLC12A6* A-allele. DNA methylation is a continuous process that allows gene expression levels to change in specific cells at a specific time, especially during critical periods of development. Potentially, all genes could be expressed in all cells at any point of time. However, gene function and regulation is modulated by genetic and epigenetic factors in a temporal and tissue-specific manner. Epigenetic mechanisms allow short-term adaptation of genomic DNA and cells to the local environment (Hartl and Jones 2001). In this context, it is noteworthy that the mature adult nervous system, composed of 10^{15} synaptic connections, requires (epi-) genetic and environmental factors, and interactions for proper organization and stabilization (Tasman et al. 2003). Recent research showed that epigenetic alterations were found to be highly associated with schizophrenia, thus also explaining discordance between monozygotic twins (Petronis 2003; Petronis et al. 2003). Interestingly, in several linkage studies, the suspected chromosomal locations for susceptibility to schizophrenia and mood disorders are compatible with genetic loci for DNA-methyltransferases and methylated

domain DNA binding proteins (DNMT1; DNMT2; DNMT3B; DNMT3L; MBD1; MBD2 and MDB4), which contribute to DNA methylation and chromatin organization (Badenhop et al. 2002; Berrettini 2001; Faraone et al. 1998; Maziade et al. 2001; Nurnberger and Foroud 2000). Additionally, alteration in only a few CpG methylation sites in *REELN*, *5HT2RA* and *MB-COMT* showed high association with psychiatric disorders (Abdolmaleky et al. 2006; Abdolmaleky et al. 2005; Abdolmaleky et al. 2004a).

The second study, which inspired this thesis, provided the first evidence that regulation of gene expression can be influenced by environmental factors. Michael Meaney's group (Weaver et al. 2004) showed that maternal care influences glucocorticoid receptor (*Nr3c1;* Nuclear Receptor Subfamily 3, Group C, Member 1) promoter methylation pattern in a rodent model. A specific type of mother-pup interaction during the first 10 days of life was found to affect the offspring's behavior mediated by the *Nr3c1* promoter methylation pattern. High maternal care was associated with low methylation of the *Nr3c1* promoter, less fearfulness and reduced hypothalamic-pituitary-adrenal axis (HPA axis) responses to stress in the offspring. Low maternal care, on the other hand, resulted in high *Nr3c1* promoter methylation and enhanced stress response. The HPA axis is a stress responsive system, which mediates the endocrine stress response, a system being under tight self-regulating control through negative feedback mechanisms, mediated by the glucocorticoid receptor. Furthermore, almost all mental disorders have been shown to be associated with dysregulation of the HPA axis, thus pointing to the glucocorticoid receptor as an interesting candidate gene in the investigation of stress related psychiatric disorders.

As the existence of different *Nr3c1* promoter epigenotypes, dependent on early environment associated with behavior and neuroendocrine alterations in adult life could be shown in rodents, we aimed to investigate whether similar regulation pattern also exist in humans.

The regulation of the HPA axis in response to acute and chronic stress is a crucial mediator of stress exposure and disease processes. As the glucocorticoid receptor is a pivotal mediator of the HPA axis, much work during the past decades has been done in order to identify mutations or SNPs located in the *NR3C1* in association with diseases. In a recent study, association of a common *NR3C1* promoter SNP (rs10482605) was demonstrated to be associated with MDD (van West et al. 2006). As regulation of the *NR3C1* expression is likely to be important for HPA axis regulation, the functional consequences of the promoter polymorphism on *NR3C1* promoter activity were investigated.

1.2 Psychiatric disorders

Mental disorders such as bipolar disorders and major depression are complex in their etiology as biological, social and psychological factors interact in the course of their development. The American Psychiatric Association (1994) defines bipolar disorders as a group of affective illnesses characterized by excessive mood shifts. Patients with BPD experience episodes of either mania (bipolar I disorder) or hypomania (bipolar II). The manic phase is characterized by persistent periods of abnormal or irritable mood accompanied by symptoms such as grandiosity, excessive talkativeness, racing thoughts, and decreased sleep. Similar symptoms are observed in hypomania, but symptomatology may be shorter and is accompanied by less associated impairment. Major depression and major depressive episodes are characterized by either sad mood or loss of interest accompanied by symptoms such as changes in weight, appetite, sleep and activity, along with fatigue, excessive guilt, impaired concentration and suicidal thoughts.

Depression, schizophrenia and bipolar disorder are complex inherited diseases with a considerable genetic contribution, and much work in the last decades has been done in order to identify mutations and polymorphisms contributing to mental health or disease. Twin and

Table 1.1: Overview for candidate genes for bipolar disorder (BPD), major depression (MDD) and schizophrenia (SCZ)

Gene	*Location*	*Functional significance*	*Disorder*	*Reference*
DISC1	1q42	Neuronal structure protein	BPD; SCZ	(Millar et al. 2001)
NR3C1	5q	Glucocorticoid receptor; transcription factor	MDD	(van West et al. 2006)
SLC6A3	5p	Mediates reuptake of dopamine	BPD	(Greenwood et al. 2001)
HTR4	5q	Encodes the HT4 receptor, which influences dopamine secretion	BPD	(Ohtsuki et al. 2002)
NRG1	8q	a signaling protein that mediates cell-cell interactions	SCZ	(Stefansson et al. 2002)
GRIN1	9q	Codes for a critical NMDA receptor subunit	BPD	(Mundo et al. 2003)
DRD4	11p	Dopamine system regulates emotion and behavior	BPD	(Muglia et al. 2002)
BDNF	11p	Neuronal growth factor involved in stress and antidepressant response	BPD, MDD	(Karege et al. 2002; Sklar et al. 2002)
DRD2	11q	Dopamine system regulates emotion and behavior	BPD	(Massat et al. 2002)
TPH2	12q	Hydroxylation of tryptophan to serotonin	MDD	(Zhang et al. 2005)
DAOA	13q	Involved in glutamatergic signaling pathway	BPD; SCZ	(Hattori et al. 2003; Schumacher et al. 2004)
SLC12A6	15q13	Cation chloride cotransporter	ACCPN, BPD	(Howard et al. 2002b; Meyer et al. 2005)
SLC6A4	17q	Promoter alleles affect transcriptional efficiency of 5HTT	BPD; MDD; Neuroto-cism, Harm avoidance	(Lesch et al. 1996; Rotondo et al. 2002)
COMT	22q	Functional variants affect enzyme activity	BPD; MDD	(Lachman et al. 1996; Rotondo et al. 2002)
MLC1	22q	Cation channel	SCZ, MLC	(Meyer et al. 2001)
MAOA	Xq	Degrades dopamine, serotonin, norepinephrin	BPD, MDD	(Preisig et al. 2000)

BDNF - brain-derived neurotrophic factor; COMT – catechol-O-methyl transferase; DAT - dopamine transporter; *DISC1* - disrupted in schizophrenia 1; *DRD* - dopamine receptor D; *GRIN1* - glutamate receptor, ionotropic, N-methyl D-aspartate 1; *DAOA* - D-amino acid oxidase activator ; *MAOA* - monoamin oxidase; *MLC1* - megalencephalic leukoencephalopathy with subcortical cysts; *NRG1* – Neuregulin 1; *SLC6A3 – solute carrier family 6 (neurotransmitter transporter, dopamine), member 3; SLC6A4* - serotonin transporter; *TPH2* - tryptophan hydroxylase 2; *HTR* - 5-hydroxytryptamine (serotonin) receptor

adoption studies indicate high heritability in these disorders (Craddock et al. 2005; Craddock et al. 2006). Linkage studies have shown that almost all chromosomes where found to harbour susceptibility genes for psychiatric diseases. A large number of genes, possibly contributing to the diseases have been identified (Table 1.1), but to date association studies are characterized by non-replication, small effect size and heterogeneity (Petronis 2004), and still no single specific gene has been identified as responsible for any major psychiatric disorder. However, investigating large pedigrees, mutations (Meyer et al. 2001; Stefansson et al. 2002), chromosomal aberrations (Millar et al. 2001), and rare polymorphisms (Meyer et al. 2005) in the genes encoding *MLC1*, *NRG1*, *DISC1* and *SLC12A6*, have been reported as causative for severe psychiatric disorders in some families, but it still has to be elucidated whether these DNA variations are located in susceptibility or in modifier genes.

There is a growing body of evidence suggesting that the understanding of the genetic etiology of psychiatric illnesses, including schizophrenia, will be more successful with integrative approaches considering both genetic and epigenetic factors. One intriguing possibility is that certain life events may interact with brain vulnerabilities created by epigenetic modification of particular genetic regions. Several mechanisms have been proposed for the development of psychiatric diseases, such as the monoamine model of mood disorder (Schildkraut 1974; Schildkraut and Mooney 2004), the neurotrophic model of depression (Duman and Monteggia 2006), mitochondrial dysfunction (Konradi et al. 2004), and viral infection during pregnancy (Brown 2006; Brown et al. 2004). There is also substantial evidence that hyperactivity of the HPA axis is involved in the pathogenesis of mood disorders (Pariante and Miller 2001). Reduced *NR3C1* expression in the frontal cortex of bipolar, major depressive, and schizophrenic patients has been described (Webster et al. 2002), providing evidence that HPA-dysregulation is present in these disorders. Elevated plasma glucocorticoid levels as seen in major depressive disorder, post traumatic stress disorder, Cushing's disease, and also in the phase of a first psychotic episode are often accompanied by hippocampal volume

reductions (Lawrie et al. 1999; Pantelis et al. 2003). This also reflects the glial deficit observed in major depressive disorder, bipolar disorder and possibly schizophrenia (Crossin et al. 1997). In vitro investigations have shown that high levels of glucocorticoid hormones result in reduced neuronal volume and dendritic arborisation (Sapolsky 2000), a phenomenon also apparent in schizophrenia and depression.

Altered HPA axis physiology and dysfunction has been consistently found in individuals with major depression. A disturbed negative feedback regulation of the HPA axis is often found in psychiatric patients including patients with schizophrenia, mania, and major depressive disorder compared to healthy controls. There is evidence that maternal stress during pregnancy has long-term neurodevelopmental effects on the maturation of the infant's brain and thus is a predictor for the development of psychiatric disorders in the adulthood. In this context, it is noteworthy that *SLC12A6*, which is predominantly expressed in the brain, is suggested to play an important role in cell growth and in the physiology of myelinisation (Adragna et al. 2004). Furthermore, rare variants in *SLC12A6* were found to represent a risk factor for bipolar disorder (Meyer et al. 2005). One intriguing possibility is that certain life events may interact with brain vulnerabilities created by epigenetic modification of particular genetic regions.

1.3 The potassium chloride cotransporter 3 (*KCC3; SLC12A6*)

The potassium chloride cotransporter 3 belongs to the cation solute carrier family 12 of ion channels (*SLC12*) consistent of 4 members (*SLC12A4-7*; also known as *KCC1-4*). The corresponding proteins are involved in the electrochemically neutral transport of ions (K^+/Cl^-) across the membrane, at which the cotransport of K^+ and Cl^- is interdependent with a 1:1 stoichiometry and low affinity constants for both ions. Members of the SLC12 protein family regulate cell volume, transepithelial salt absorption, renal K^+ secretion, myocardial K^+ loss during ischemia and neuronal Cl^- concentration, thus modulating neurotransmission.

SLC12A6 was identified simultaneously by three independent groups (Hiki et al. 1999; Mount et al. 1999; Race et al. 1999). The gene, which is arranged of 26 exons, is located on chromosome 15q13-14 and expression was shown in multiple tissues including brain, kidney, heart and liver. Various *SLC12A6* transcripts have been described so far, mostly due to alternative first exon usage with transcriptional initiation at separate promoters. Initially, the first identified isoforms were nominated *KCC3a* (expressed mainly in brain, kidney, muscle and heart) and *KCC3b* (most abundant in kidney). The longer 1150 amino acid (aa) SLC12A6 isoform (*KCC3a; SLC12A6-1*) encodes 51aa not present in the 1099aa KCC3b variant. Within this 51aa in the NH2-terminal region, seven potential phosphorylation sites for protein kinase C (PKC) are present, suggesting different posttranslational regulation of KCC3a and KCC3b.

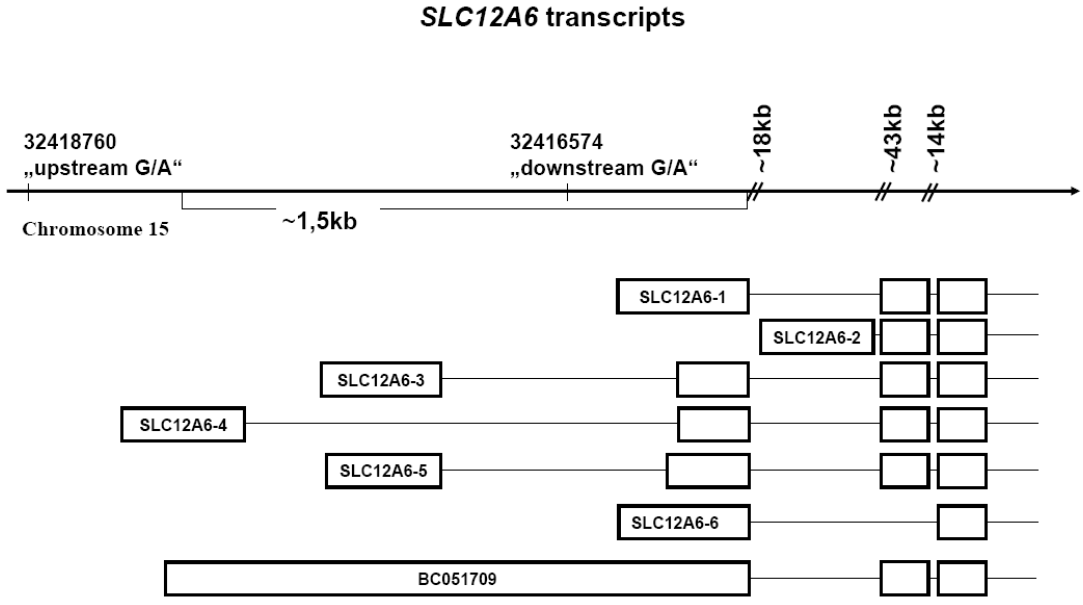

Figure 1.1: Alternative *SLC12A6* transcripts and splice variants as described by Mercado et al. (2005) are illustrated, and aligned to the respective chromosomal position on chromosome 15; “upstream and downstream SNPs” investigated in the study are indicated with respect to their localisation on chromosome 15.

In the meantime, six *SLC12A6* alternative transcripts have been identified (Mercado et al. 2005), in which *SLC12A6-1+3-6* share the same 5' regulatory region, whereas *SLC12A6-2* is transcribed from a promoter located approximately 18 kb 3' of *SLC12A6-1* (Figure 1.1). *SLC12A6-1,* and with high probability all *SLC12A6* transcripts under control of the upstream promoter, are expressed in most brain areas, including hypothalamus, cerebellum, brainstem, cerebral cortex, and white matter. It is also abundant in spinal cord and is expressed at very low levels in dorsal root ganglia (Le Rouzic et al. 2006; Mercado et al. 2004; Mercado et al. 2005). *SLC12A6-1* expression correlates with development of myelin, because expression is low at birth and increases during postnatal development, similar to the pattern observed for myelin-binding protein (Pearson et al. 2001).

Mutations and polymorphisms in *SLC12A6* were shown to be causative for the Andermann Syndrome and to be associated with BPD (Table 1.2). Andermann Syndrome is an autosomal recessive motor and sensory neuropathy with agenesis of the *corpus callosum* associated with developmental and neurodegenerative defects and dysmorphic features (Howard et al. 2002a; Howard et al. 2003; Howard et al. 2002b). Interestingly, some of the clinical manifestations of the Andermann Syndrome are due to altered neuronal development, while others are due to neurodegeneration - particularly in white matter. The clinical picture includes deficiencies of both, central and peripheral nervous system as well as cognitive activity. Most of the neurological problems seen in Andermann's disease also are apparent in *Slc12a6* knockout mice (Boettger et al. 2003; Howard et al. 2002b). In 2005, after performance of linkage analysis two rare G/A SNPs (G/A upstream at position 32418760 and G/A downstream at position 32416574 on chromosome 15) located in the *SLC12A6* 5' region showing high association with BPD were identified. The role of *SLC12A6-1* in neuronal maturation and the fact that the upstream SNP represents a position for epigenetic modification potentially triggered by external signals led to the functional *SLC12A6* promoter investigation described

in chapter 3, which combines the study of single nucleotide polymorphisms with epigenetic modification.

Table 1.2: *SLC12A6* mutations and polymorphisms with associated phenotypes

Phenotype	***Underlying SLC12A6 sequence variation***	***Reference***
ACCPN	exon 18 at nucleotide 2436 (2436delG); thr813fsX813	(Howard et al. 2002b)
ACCPN	1 guanine, at positions 1584 and 1585 truncate 619 amino acids from the SLC12A6 protein (phe529fsX532).	(Howard et al. 2002b)
ACCPN	3031C-T transition in exon 22 of the *SLC12A6* gene, resulting in an arg1011-to-ter (R1011X)	(Howard et al. 2002b)
ACCPN	2023C-T transition in exon 15 of the *SLC12A6* gene, predicted to result in an arg675-to-ter (R675X)	(Howard et al. 2002b)
BPD; periodic catatonia	compound heterozygosity of 2 A/G SNPs in the 5' regulatory region	(Meyer et al. 2005)
ACCPN	1-bp insertion (2031insT) and an 8-bp deletion (1478delTTCCCTCT), both are predicted to result in a frameshift	(Uyanik et al. 2006)
ACCPN	1-bp deletion (901delA) in the *SLC12A6* gene, resulting in premature truncation of the protein at codon 315	(Uyanik et al. 2006)
ACCPN	homozygous 619C-T transition in the *SLC12A6* gene, resulting in an arg207-to-cys (R207C) substitution	(Uyanik et al. 2006)
ACCPN	10-bp deletion (nucleotides 2994-3003) in exon 22 of *SLC12A6*	(Salin-Cantegrel et al. 2007)
Deafness, locomotor deficits; severe central and peripheral neurodegeneration; sensorimotor gating defects	*Slc12a6* knockout	(Boettger et al. 2003; Howard et al. 2002b)

The evidence that non-functionality of *SLC12A6* is associated with mental diseases and the results of linkage and association studies that supported the importance to investigate the functionality of the promoter variants identified by Meyer et al. (2005), laid out the rationale of the study as described in chapter 2. Furthermore, as the variants are located in the *SLC12A6 5'* regulatory region, it was hypothesised that promoter activity could be modulated by a

combination of altered allelic epigenetic and DNA primary structure, thus potentially linking DNA variation with epigenetic modification.

1.4 Regulation of the HPA

The HPA axis is a central regulatory system of the organism, which evolved to maintain a dynamic equilibrium, called homeostasis. At any time, this equilibrium is threatened by external, physical as well as psychological events defined as "stressors".

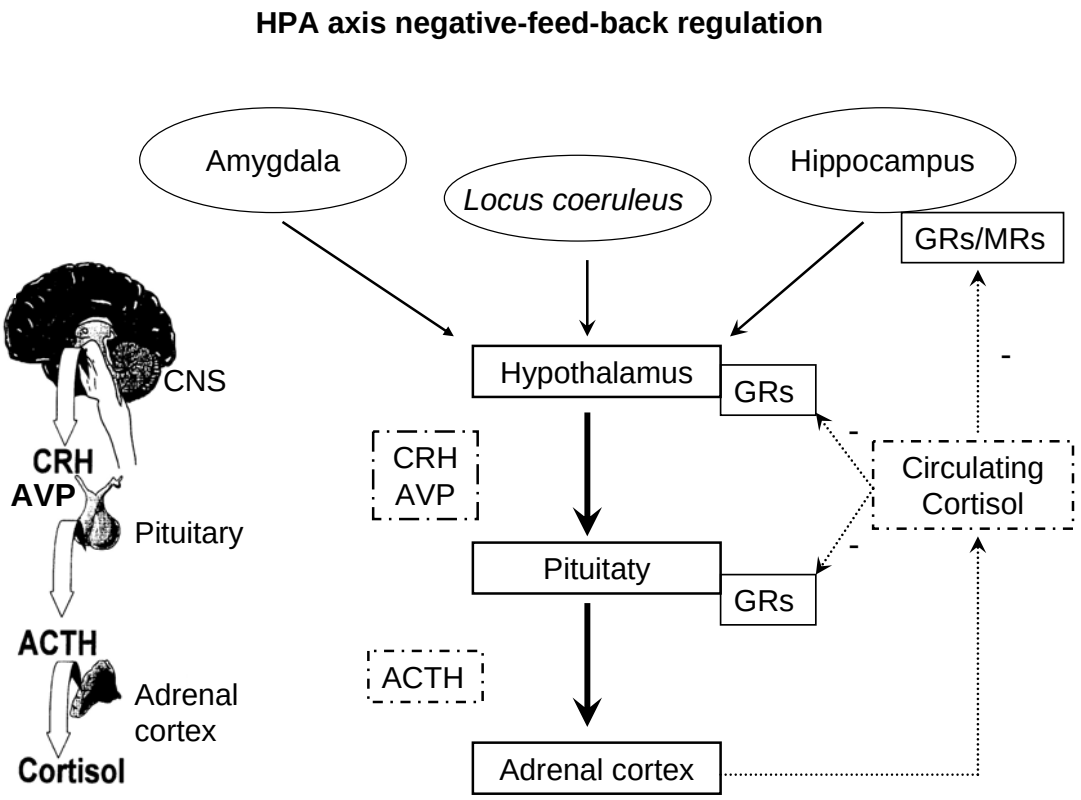

Figure 1.2: Schematic diagram of the HPA axis, describing regulation and negative feedback (-) of cortisol via glucocorticoid receptors (modified, according to Juruena et al. 2004).

In response to stress, the brain activates a hierarchical hormone system consistent of the hypothalamus, the pituitary gland, and the adrenal cortex. Under complex regulation

originating from hippocampus, amygdala and *locus coeruleus*, corticotropin releasing hormone (CRH) and vasopressin (AVP) are secreted by the paraventricular nucleus (PVN) of the hypothalamus in face of external or internal challenge. After binding to its specific receptor at the anterior pituitary, CRH leads to cleaveage of pro-opiomelanocortin (POMC) into adrenocorticotropic hormone (ACTH), beta-endorphin, alpha-, beta-, and gamma melanocyte- stimulating hormone (MSH) besides others. After release, ACTH is transported by the bloodstream to the adrenals where it triggers glucocorticoid release (cortisol in humans and corticosterone in rats) from the adrenal cortex.

Free corticosteroids (90-95% in blood is bound to corticosteroid binding globuline; CBG), feed back to the HPA system at the level of the PVN and the anterior pituitary to suppress CRH and ACTH release, respectively (Jacobson and Sapolsky 1991; Mendel 1989; Mendel et al. 1989). Overall HPA reactivity is controlled by several negative feedback loops, regulated by mineral- and glucocorticoid receptors (de Kloet et al. 1998). The glucocorticoid receptor mediates most of the hormone-induced actions after hormone binding in the cytoplasm and translocation into the nucleus. Furthermore, glucocorticoids have a wide range of physiological effects via genomic as well as nongenomic pathways. After binding to their receptors, glucocorticoids exert their function in order to mobilize energy, suppress immune function, and to potentiate sympathetic nervous stimuli (Sapolsky et al. 2000). As the glucocorticoid receptor is expressed ubiquitously, dysregulation of the HPA axis is associated or even causative for the onset of a multitude of psychosomatic and psychiatric diseases (Chrousos and Gold 1992; Holsboer 1989).

1.4.1 The glucocorticoid receptor (NR3C1)

The *NR3C1* gene, which is organized in 9 exons, is located on chromosome 5q31-32 spanning a 140 kb region (Lu and Cidlowski 2006). It contains 8 coding exons (exon 2-9), and to date 11 alternative first exons have been identified, representing solely the 5’-untranslated region

(Presul et al. 2007; Turner and Muller 2005). The 1197 bp exon 2 encodes most of the protein's N-terminus including the transactivation domain. Exons 3 and 4 (167 bp; 117 bp) encode the two zinc-finger domains involved in DNA-binding. Exons 5-9alpha/beta represent the ligand-binding domain as well as the 3'-untranslated region.

Since individual differences in HPA axis stress responses appear to be highly associated with psychiatric diseases, the investigation of the genetic basis possibly underlying these phenomena on the level of *NR3C1* regulation (as described in chapter 3 and 4) is of major interest.

1.4.2 From maternal separation and early handling to epigenetic programming

Stress reactivity in the adulthood is modulated by genetic background as well as early experience. In his pioneering studies, Seymour Levine could show that maternal separation in rats led to lifelong reduced emotional and neuroendocrine reactivity to common stressors in the offspring (Dent et al. 2000; Levine 1957; Levine et al. 1957). Investigators could demonstrate that, additionally to the individual's genetic make-up early life experience, such as pre-/ peri- and postnatal mother-offspring interaction, predicts health outcome. More recently, Michael Meaney's group have found correlations between the early experienced environment with the offspring's behavior in adulthood. They identified a high licking and grooming and arched back nursing- compared to a low licking and grooming and arched back nursing maternal behavior to being correlated with attenuated stress-system activity, improved cognitive performance in a spatial learning test and reduced anxiety-like behavior in adulthood. Moreover, they could identify physiological, genetic and epigenetic correlates for behavioral mechanisms dependent on the experienced early environment. Postnatal handling as well as maternal care increase glucocorticoid receptor expression in the hippocampus (Meaney et al. 1993; Meaney et al. 1994; O'Donnell et al. 1994), a region that has been strongly implicated as a critical site for glucocorticoid negative feedback regulation. It could

be shown that triiodthyronin-mediated, postnatal handling increases 5HT turnover in hippocampus and frontal cortex (Mitchell et al. 1990a; Mitchell et al. 1990b; Smythe et al. 1994a; Smythe et al. 1994b). Interestingly, these regions are showing altered glucocorticoid receptor expression after early handling, whereas it remains unaltered in hypothalamus and amygdala (Smythe et al. 1994a; Smythe et al. 1994b). As illustrated in Figure 3.1, binding of serotonin to its specific HT_7 receptor leads to enhanced cAMP turnover. Consequently, protein kinase A (PKA) is activated and phosphorylates the transcription factors activator-protein 2 (Ap-2), cAMP response element binding protein (Creb), and nerve growth factor inducible factor-a (Ngfi-a). Additionally, the expression of these transcription factors in hippocampus is enhanced after early handling, and the glucocorticoid receptor gene promoter harbours numerous binding sites for these transcription factors (Meaney et al. 2000). The characterization of at least eleven alternative *Nr3c1* mRNAs cloned from rat hippocampus (McCormick et al. 2000), differing in the usage of alternative first exons most of them under control of its own promoter further stimulated *Nr3c1* promoter investigation. Whereas two alternative first exons specific transcripts were found being expressed in all tissues investigated (exon 1_6 and exon 1_{10}), exons 1_5-, 1_7, and 1_{11} *Nr3c1*-specific transcripts showed significant levels in hippocampus and were expressed at either low or undetectable levels in other tissues. Interestingly, in CNS cells, *Nr3c1* alternative first exon specific promoter constructs revealed highest luciferase expression for the exon 1_7 specific construct. Additionally, mRNA containing exon 1_7 in hippocampus was selectively elevated by postnatal handling. In the first studies, Ap-2 and Ngfi-a binding to *Nr3c1* promoter sequences was demonstrated (Meaney et al. 2000). The epigenetic mechanism of maternal programming for the first time was described in 2004, and Ngfi-a was identified as the critical transcription factor mediating environment with cellular adaptation (Weaver et al. 2004). In their study, Weaver and colleagues (2004) described HPA responsiveness in rodents as a result of early life experience mediated by DNA (de-) methylation and histone (de-) acetylation. According

to Michael Meaney's group, the offspring of high licking and grooming mothers had higher hippocampal *Nr3c1* expression compared to the offspring of low caring mothers. They could show that this effect is mediated through differences in exon 1_7 specific promoter methylation, histone acetylation and Ngfi-a binding mediated in dependence of experienced maternal care. According to their study, high maternal care leads to low *Nr3c1* 1_7 promoter DNA methylation, histone acetylation and enhanced Ngfi-a binding, whereas low maternal care leads to high *Nr3c1* 1_7 specific promoter DNA methylation accompanied by histone deacetylation and low Ngfi-a binding. Furthermore, they could show that the epigenetic profile is stable life-long, can be transmitted non-genetically to the next generation, is reversed by cross-fostering, and can be modulated pharmacologically by trichostatin A (TSA) treatment (Weaver et al. 2004; Weaver et al. 2005). In this context, the identification of the 5' organization of the human *NR3C1* as described in 2005 (Turner and Muller 2005) was more than welcomed. Similarly to the situation also described for *SLC12A6*, and highly orthologous to the rat 5' *Nr3c1* organization, alternative first exon usage also was found for *NR3C1* (Figure 1.3).

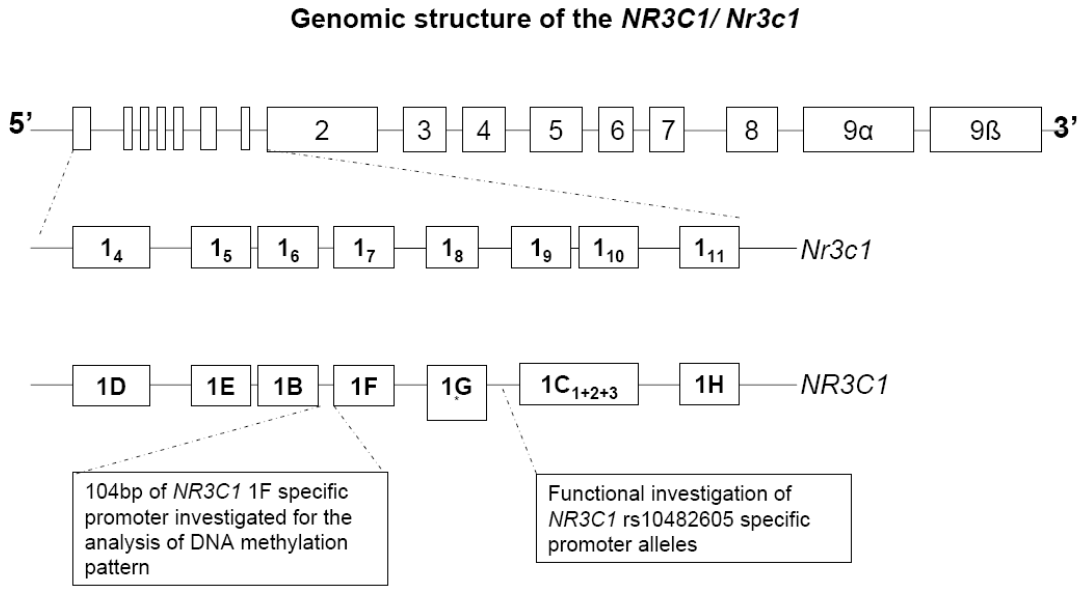

Figure 1.3: *Nr3c1* and *NR3C1* genomic regions with respect to the 5' *NR3C1* regulatory genomic regions investigated in the studies

1.5 Epigenetics / Chromatin modifications

The term epigenetic was introduced by Conrad Waddington in 1942 to describe "The interactions of genes with their environment that bring the phenotype into being" (Waddington, 1942). Currently this term is widely used in order to explain cell type-specific regulation of gene expression. Epigenetics in general is the study of cellular mechanisms, which control somatically heritable gene expression states without any change in the underlying DNA sequence (Bird 2002). Epigenetic mechanisms include DNA methylation, histone modifications such as acetylation and methylation, and control of mRNA expression by non-coding RNAs (Bird 2002; Jaenisch and Bird 2003). DNA methylation is achieved by a class of proteins named DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) onto the 5' position of the cytosine ring residing in most cases at the dinucleotide sequence CG. To date, three different DNA methyltransferases have been described such as DNMT1, which is a maintenance methyltransferase using hemimethylated DNA as a substrate, and DNMT3a and b which are *de novo* methyltransferases. Tissue and developmental specific methylation pattern have been described, accomplished by the interaction of the different DNMTs.

DNA methylation silences genes by two principle mechanisms. First, CpG methylation inhibits binding of transcription factors, for example as shown for Ngfi-a (Meaney et al. 2000; Weaver et al. 2004) or MYC/MAC (Prendergast and Ziff 1991) and second, methylated DNA recruits methylcytosin DNA-binding proteins such as MECP2 to the gene. These DNA binding proteins recruit chromatin modification enzymes such as histone deacetylases (HDACs) and histone methyltransferases (HMTs), which in turn introduce silencing of chromatin.

The paradigm of a stable DNA methylation pattern formed during embryogenesis and maintained thereafter by maintenance DNA methyltransferase 1, as shown in imprinted genes like *IGF2* or as observed in X-chromosome inactivation, dominated the thinking of most

scientists until the beginning of this century. However, the existence of different epialleles in humans was demonstrated during the past decade. Furthermore, epigenetic differences were found in monozygotic twins that were discordant for schizophrenia or Beckwidth-Wiedemann syndrome (Petronis et al. 2003; Weksberg et al. 2002). Interestingly observed epiallelic differences in MZ twins were the more apparent, the more the inter-individual life styles differed (Fraga et al. 2005). Additionally, the epigenome was demonstrated to be modified by external factors such as development, nutrition, medication, early experiences and/or environment in general.

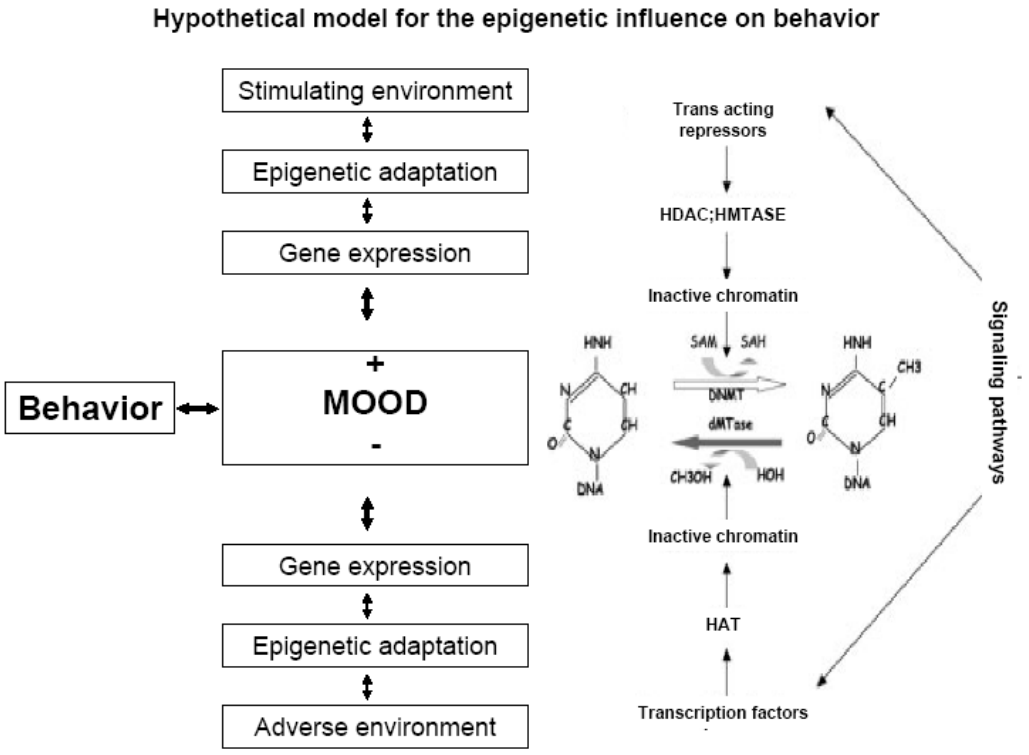

Figure 1.4: Different environmental factors trigger DNA methylation pattern. Active chromatin facilitates DNA demethylation while inactive chromatin facilitates DNA methylation - both mechanism are discussed to influence behavior (modified, according to Szyf et al. 2008).

The theory that epigenetic mechanisms may be associated with mental diseases (Petronis et al. 2000) was initiated by the findings that two neurodevelopmental disorders, Angelman- (AS)

and Prader-Willi Syndrome are caused by disturbed genetic imprinting. Mutations in *MECP2* leading to an unfunctional MECP2, a nuclear protein of crucial importance in the epigenetic control of imprinted genes are responsible for Rett syndrome in 80% of cases. Furthermore, a number of epimutations in the genes for *MB-COMT*, *RELN*, *5HT2R* were shown to be associated with mental diseases, such as BPD, MDD, SCZ and autism spectrum disorder (ASD; Abdolmaleky et al. 2006; Abdolmaleky et al. 2005; Abdolmaleky et al. 2004b; Costa et al. 2002; Grayson et al. 2006; Grayson et al. 2005; Mill et al. 2008; Petronis et al. 2000).

1.6 Genetic polymorphisms vs. epigenetic modifications

Gene expression can be modulated by genetic and/or epigenetic mechanisms. DNA polymorphisms or *de novo* germ line mutations result in the same genetic change in all cells of the particular organism. In these cases, change in the DNA primary structure is inert and unique in the whole organism. However, somatic alterations in DNA methylation pattern occur cell specifically and were shown to be modifiable by environmental influences. Inhibitors of DNA methylation such as 5-azacytidine (5-aza; a chemical analogue of cytidine) or HDAC inhibitors like trichostatin A (TSA) can lead to DNA demethylation and (re-) activation of genes originally silenced in the cell. As it was shown in cancer research, aberrant silencing of tumor suppressor genes by DNA methylation can cause severe illness. Therefore, it can be speculated that alternative DNA methylation also could affect expression of behavior-regulating genes in the brain. A specific DNA methylation pattern in the brain might occur as a consequence of either pathological or adaptive physiological mechanisms. It should also be considered that altered epigenetic states are not only the effect of different behavioral exposures - they could also affect behavior. This suggests a loop through which social exposure affects epigenetic states. These epigenetic states in turn could affect social behavior as well as behavioral pathologies.

During the past decade, mostly two basic approaches to identify susceptibility genes for complex disorders were used - linkage and association studies. Linkage studies are a hypothesis-neutral search for markers that co-segregate with a disease, followed by fine mapping and positional cloning to identify a gene or a polymorphism responsible for a certain phenotype or disease. Association studies are used to investigate whether particular variants of a candidate gene are more prevalent in patients compared to healthy controls.

The studies presented here are combining the benefits of linkage and association studies with epigenetic and functional analysis of *SLC12A6* and *NR3C1* promoter elements. As cellular mechanisms underlying psychopathology are thought to develop over time or under stress related conditions, it was self evident to investigate promoter methylation and regulation as these genomic regions dynamically influence gene transcription also subjected to environmental stimuli.

This **Chapter 1** is intended to present and explain the basic ideas this thesis was based on. Furthermore, the theoretical background on *SLC12A6* and *NR3C1*, their genetic organization as well as their physiological roles should be highlighted. Additionally, this Chapter is intended to give an overview on the existing literature on single nucleotide polymorphisms and DNA methylation in the pathogenesis of psychiatric disorders. In **Chapter 2,** own findings on the functionality of *SLC12A6* promoter polymorphisms, associated with bipolar disorders will be presented. Hippocampal *NR3C1* promoter methylation pattern as investigated in 32 postmortem human brain tissues will be elucidated in **Chapter 3**, followed by a study presenting functional analysis of a *NR3C1* promoter variant that has shown association with major depression in **Chapter 4**.

In **Chapter 5,** the results will be discussed against the background of existing literature followed by an outlook delineating future research directions in **Chapter 6**.

Chapters 2-4 represent manuscripts on different topics which are already published (Chapter 2 and 3) or are accepted for publication (Chapter 4) in scientific journals. Therefore, they also can be read separately, but also making a certain amount of redundancy unavoidable. Contribution of collaborating colleagues to the respective manuscript is reflected by the author list ranking indicated at the beginning of each chapter.

CHAPTER 2

Functional analysis of a potassium-chloride cotransporter 3 (*SLC12A6*) promoter polymorphism leading to an additional DNA methylation site

Dirk Moser, Savira Ekawardhani, Robert Kumsta, Haukur Palmason, Christoph Bock, Zoi Athanassidou, Klaus-Peter Lesch & Jobst Meyer

2.1 Abstract

The human potassium-chloride co-transporter 3 (*KCC3, SLC12A6*) is involved in cell proliferation and in electro-neutral movement of ions across the cell membrane. The gene (*SLC12A6*) is located on chromosome 15q14, a region that has previously shown linkage with bipolar disorder, schizophrenia, rolandic epilepsy, idiopathic generalized epilepsy (IGE), autism and attention deficit/hyperactivity disorder (ADHD). Furthermore, recessively inherited mutations of *SLC12A6* cause Andermann syndrome, characterized by agenesis of the *corpus callosum*, which is associated with peripheral neuropathy and psychoses. Recently, we have demonstrated the association of two G/A promoter polymorphisms of *SLC12A6* with bipolar disorder in a case-control study, and familial segregation of the rare variants as well as a trend towards association with schizophrenia.

In order to investigate functional consequences of these polymorphisms, lymphocyte DNA was extracted, bisulfite modified and subsequently sequenced. To investigate *SLC12A6* promoter activity, various promoter constructs were generated and analyzed by luciferase reporter gene assays.

We provide evidence that the G- allele showed a significant reduction of reporter gene expression. In human lymphocytes, the allele harbouring the rare upstream G nucleotide was found to be methylated at the adjacent C position, possibly accountable for tissue specific reduction in gene expression *in vivo*.

Here we demonstrate functionality of a SNP associated with psychiatric disease and our results may represent a functional link between genetic variation and an epigenetic modification.

2.2 Introduction

The potassium-chloride co-transporter 3 (*SLC12A6*, formerly *KCC3*; OMIM %604878) belongs to a protein family consisting of four identified transporters so far (*SLC12A4-7; KCC1-4*). These transporters are involved in cell proliferation (Shen et al. 2001) and in the electro-neutral movement of ions across the plasma membrane, thus controlling ionic and osmotic homeostasis of various cell types. The gene *SLC12A6* is located on chromosome 15q13-14 (Hiki et al. 1999; Mount et al. 1999; Race et al. 1999). Several groups have demonstrated that mutations in *SLC12A6* are causative for the recessively inherited Andermann syndrome (ACCPN, OMIM %218000), a severe neurological disorder characterized by agenesis of the *corpus callosum*, peripheral neuropathy (Howard et al. 2003; Howard et al. 2002b; Uyanik et al. 2006) and psychoses (Filteau et al. 1991). The chromosomal region 15q13-14 represents a shared susceptibility locus for a variety of neuropsychiatric disorders including periodic catatonia, bipolar disorder, schizophrenia, rolandic epilepsy, idiopathic generalized epilepsy (IGE), autism and attention deficit/hyperactivity disorder (ADHD; Elmslie 1997a; Elmslie et al. 1997; Elmslie 1997b; Sander et al. 1997).

Interestingly, approximately 1% of the total pool of human genes are coding for solute carrier genes (Gamba 2005). The genes encoding cation-Cl^- coupled co-transporters are grouped together in the SLC12 family. These co-transporters have recently gained attention in the field of neuroscience as they facilitate neuronal response to gamma-aminobutyric acid (GABA) and glycine (Mercado et al. 2004). Furthermore, several members of the cation-chloride co-transporter family are involved in hereditary diseases such as Gitelman`s, Bartter`s, Gordon`s and Andermann`s syndromes, and have shown associations with bipolar disorder (Bianchetti et al. 1992; Dupre et al. 2003; Filteau et al. 1991; Gamba 2005; Gitelman et al. 1966; Gordon 1986; Howard et al. 2003; Howard et al. 2002b; Meyer et al. 2005; Uyanik et al. 2006). The crucial role of cation co-

transporters for neuronal development is also supported by animal studies in which knockout mice for cation-chloride co-transporters display a plethora of symptoms such as deafness, locomotor deficits, severe central and peripheral neurodegeneration, and sensorimotor gating defects (Boettger et al. 2002; Boettger et al. 2003; Hubner et al. 2001).

Hence, *SLC12A6* may also be regarded as a valuable candidate gene for complex inherited psychiatric disorders. Initially, two first exons denoted *SLC12A6-1* and *SLC12A6-2* (formerly *KCC3a* and *KCC3b*) which are under the control of separate promoters (Di Fulvio et al. 2001; Mercado et al. 2004), have been described where the transcript possessing alternative exon 1a is predominantly expressed in brain. So far, 6 alternative transcripts have been identified for *SLC12A6* (Mercado et al. 2005). All transcripts except for *SLC12A6-2*, are regulated by the same promoter.

Recently, we have reported two G/A polymorphisms in the *SLC12A6* 5' regulatory region denoted as 32418760 G/A and 32416574 G/A (numbering of nucleotide positions are relative to chromosome 15 of the UCSC hg18 assembly of the human genome, March 2006; no rs numbers available). These alleles are consecutively referred to as “upstream G/A” (nt 32418760) and “downstream G/A” (nt 32416574). Both G variants are rare, with frequencies of about 6 %, and in strong linkage disequilibrium with each other. They were found to be significantly associated with bipolar disorder in a case-control study. Furthermore, in a large family with seven affected members, the rare variants were co-segregating with periodic catatonia (OMIM %605419), a subtype of unsystematic schizophrenia according to Leonhard’s classification (Meyer et al. 2005).

Apart from investigating genetic variations as risk factors for psychiatric disease, the study of epigenetic mechanisms is gaining growing importance in the field of neuropsychiatry. Epigenetics comprises all cellular mechanisms that are involved in the

"structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Jaenisch and Bird 2003). The most prominent mechanisms of epigenetic gene regulation are DNA methylation (Jaenisch and Bird 2003; Rakyan and Beck 2006) and histone modifications. It could be demonstrated that differences in epigenetic states such as differential CpG methylation are affected by environmental factors, including maternal care and critical life events (Fraga et al. 2005; Weaver et al. 2004; Weaver et al. 2005). It was also demonstrated that DNA methylation patterns at specific genes are associated with bipolar disorders, schizophrenia and major depression (Abdolmaleky et al. 2006; Grayson et al. 2005). Studies of the reelin gene- (*RELN*) and the catechol-O-methyltransferase gene (*COMT*) promoter identified key bases that function in both positive and negative transcriptional regulation depending on their DNA methylation status. In addition, the methylation pattern of these *cis*-acting elements are significantly different between schizophrenic and non-schizophrenic subjects (Abdolmaleky et al. 2005; Abdolmaleky et al. 2004; Grayson et al. 2006; Grayson et al. 2005).

The aim of the present study is to investigate the functionality of the *SLC12A6* promoter variants. Since cytosines which are directly followed by a guanine are target for DNA methylation, we hypothesized that the presence of the G nucleotide at the upstream G/A polymorphic site could lead to methylation of the adjacent C *in vivo*. We thus analyzed the DNA methylation state of the upstream CpG site in affect patients and tested *in vitro* methylated *SLC12A6* promoter constructs carrying the G allele or the A allele for their activity in luciferase reporter gene assays.

2.3 Materials and Methods

2.3.1 Sample collection

Blood samples of all affected and unaffected members of family 11 were obtained as described before (Meyer et al. 2005; Stober et al. 2001; Stober et al. 2000), and DNA was extracted according to the salting out procedure (Miller et al. 1988).

2.3.2 Bisulfite modification

Bisulfite modification of DNA was performed as described elsewhere (Frommer et al. 1992; Grunau et al. 2001) with minor modifications. DNA was obtained from affected and unaffected members of family 11 (Meyer et al. 2005) heterozygous for both polymorphisms located in the *SLC12A6* 5' regulatory region. Two µg of genomic DNA were incubated for 20 min at 42°C in 50 µl of 0.3 M NaOH solution. Subsequently, thirty µl of 10 mM hydroquinone (Sigma), and 520 µl of freshly prepared 40.5 % sodium bisulfite solution (Sigma) at pH 5 were added to the denatured samples and incubated for 16 h at 55°C in the dark. Modified DNA was desalted using the Wizard DNA purification resin (Promega) according to the manufacturer's protocol and eluted into 50 µl of water. Modification was completed by NaOH treatment (final concentration, 0.3 M) for 10 min at room temperature followed by the addition of 10 M ammonium acetate to a final concentration of 3 M. Additionally, 10 µg of salmon sperm DNA were added as a carrier before ethanol precipitation. Finally, DNA was re-suspended in 25 µl of water and used immediately for PCR or stored at -20°C.

2.3.3 PCR amplification:

The sequence of interest in the bisulfite-treated DNA was amplified by nested PCR with primers specific for the bisulfite-treated sequence (Table 2.1). To control for efficiency

of the bisulfite modification, PCR was also performed with primers selectively targeting untreated genomic DNA.

Table 2.1: PCR Primer sequences

Region chromosome 15	**PCR primers (5'→ 3'), hybridization temperature $MgCl_2$ concentration and DMSO**	**Product length (bp)**
Accession no.: NM_133647: 32418817 - 32418400	**Forward genomic:** TGAATCAAGAAACCCAGACT **Reverse genomic:** ATTCCATGTTTTCACCACTAC 50°C 1,5 mM	418
Accession no.: NM_133647 32418842 -32418564	**Forward bisulfite** GAGGATGGATGAAAGTTGTGGG **Reverse bisulfite** CCCAAAAACTAACCCCCRAT 57°C; 1,5 mM 5 % DMSO	278
Accession no.: NM_133647 32418839 - 32418588	**Forward bisulfite nested** GATGGATGAAAGTTGTGGGTT **Reverse bisulfite nested** CCTAAAATCTTAACTCCTCACAAATAA 54,2°C; 2 mM	251
Accession no.: NM_133647 32419307 -32416464	**Forward sense*Kpn*I_for Luciferase** ggtaccGTTGGCTGCAGTTCTGCCTTTATCTTA **Reverse sense*Xho*I_rev Luciferase** ctcgagAAGAGCTACCTAGCTAACCCCTCTGGT 64°C 1,5 mM	2844
Accession no.: NM_133647 32419307 -32416464	**Forward antisenseXho_for Luciferase** ctcgagGTTGGCTGCAGTTCTGCCTTTATCTTA **Reverse antisenseKpnI_rev Luciferase** ggtaccAAGAGCTACCTAGCTAACCCCTCTGGT 64°C 1,5 mM	2844
SLC12A6 cDNA 11060569 - 11055819	**SLC12A6 cDNA specific Primer_for** CGGACATAAGAAAGCTCGAAA **SLC12A6 cDNA specific Primer_rev** CAGTCAACATTGTACAGCAGCA 58°C 2 mM	383

Bisulfite-specific PCRs were performed in a final volume of 50 µl containing 5 µl of bisulfite-modified DNA (2 µl PCR product for nested PCR; 100 ng DNA for genomic PCR respectively), 20 pmol of each primer (10 pmol of each primer for nested and

genomic PCR), 200 µM of each dNTP, 1.0 or 2.0 mM $MgCl_2$, 50 mM KCl, 10mM Tris HCl (pH 8.3 at 25°C), 0.0025 mg ml^{-1} BSA, 0.025% Tween 20 and 2,5 U High Fidelity Taq Polymerase (MBI-Fermentas). To the first bisulfite-specific PCR, 5% dimethylsulfoxide (DMSO) was added.

Amplification was performed in an ABI GeneAmp®9700 cycler: 5 min at 95°C were followed by 35 cycles, (30 sec at 95°C, 45 sec at the annealing temperature listed in table 1, and 30 sec at 72°C) ending with a final extension step at 72°C for 7 min. Controls without DNA were performed for each set of PCR`s. PCR products (10 µl) were separated on a 1.5 % agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Nested PCR products were gel-purified, fluorescence labelled (Big dye terminator kit; ABI) and directly sequenced in an ABI 310 automated sequencer (Applied Biosystems, Foster City, USA).

2.3.4 Assembly of luciferase reporter gene plasmids

Reporter gene plasmids were created by cloning four 2844 bp PCR fragments amplified from the 5'-flanking region of *SLC12A6*. Amplicons covered the chromosomal nucleotide positions nt 32419307 – 32416464 according to UCSC Browser of May, 2004. Primer-specific *Kpn*I and *Xho*I recognition sites were added to the PCR products (see Table 1.1).

The fragments were consistent with the *SLC12A6 G*- and A variant promoters, covering both upstream and downstream G/A polymorphic sites. PCR fragments were digested with *Kpn*I- and *Xho*I and ligated into the appropriate sites of pGL3-Basic luciferase reporter plasmid which was cut by the same enzymes, with both allelic sequences represented in both sense (pGl3-A-A; pGl3-G-G) and antisense directions (pGl3-A-Ainv; pGl3-G-Ginv). To distinguish between putative functional consequences of up-

and downstream polymorphic promoter sites, a 953 bp fragment (nt 32418869 - 32417927) including the upstream G/A site was deleted by using *Stu*I (see figure 2.1). Subsequently, the plasmids were re-ligated, resulting in constructs differing solely in their downstream G/A polymorphic region (pGL3-del-A; pGl3-del-G). To control for sequence integrity, all constructs were completely sequenced.

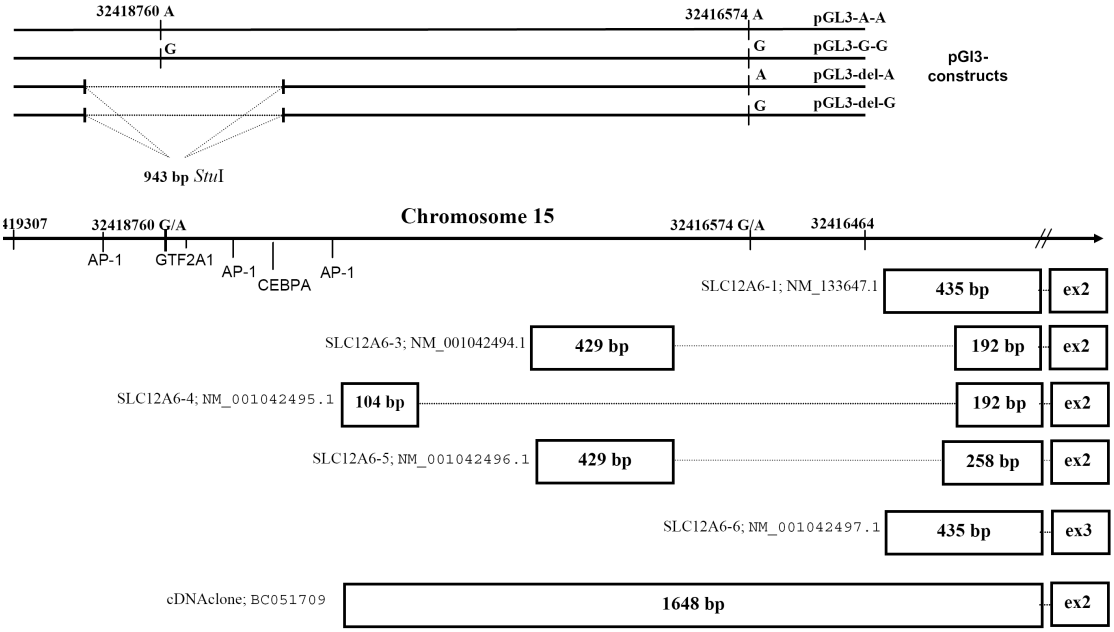

Figure 2.1: Overview of the *SLC12A6* 5' regulatory region
Overview of the *SLC12A6* 5' regulatory region. Luciferase constructs, polymorphic 32418760 G/A and 32416574 G/A nucleotides (Meyer et al. 2005) are indicated. Transcription factor binding sites, *Stu*I cutting sites, alternative *SLC12A6* transcripts (Mercado et al. 2005) and a putative new *SLC12A6* transcript (BC051709) identified by *in silico* analysis are shown according to their positions on chromosome 15.

2.3.5 In vitro methylation

SLC12A6 promoter constructs were methylated in vitro by using *Sss*I methylase and S-adenosyl methionine under conditions recommended by the manufacturer (New England Biolabs). Complete CpG methylation was ascertained by digesting the methylated DNA with an excess (20 U/mg) of restriction enzymes *BstU*I or *Tai*I. Only

completely methylated DNA preparations were used for transfection under conditions described below.

2.3.6 Transient transfection and luciferase assays

U373MG glioblastoma-astrocytoma (ECACC 89081403), *SLC12A6*-expressing cells, were grown in minimal essential Eagle`s medium (Biowest), supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 1 % penicillin/streptomycin (Pen/Strep) and 1 % non essential amino acids (NEAA) at 37°C in a humified atmosphere at 5 % CO_2. Cells were transfected by using Fugene6 transfection reagent (Boehringer) in a ratio of 2:1 (µl Fugene6 / µg DNA). Cells were passaged at ~80 % confluency and spread in a density of 1 x 10^5 cells / ml in 24 well plates. After 24 h of incubation, 0.8 µg firefly luciferase constructs with 0.2 µg pGL4.74 (HRLUC/TK, *Renilla* luciferase; Promega), 2 µl Fugene6 and 20 µl serum free medium were added to the cell culture. The plasmid expressing *Renilla* luciferase was used to control for transfection efficiency. Promoter activity was tested with and without 50 µM forskolin, 2 µM phorbol 12-myristate acetate (PMA), and 50 µM dexamethasone added 24 hours after transfection. As internal control, pGL3-control and empty pGL3-basic vectors were transfected separately. Cells were harvested in 100 µl Passive Lysis Buffer (PLB, Promega) 48 h after transfection. Additionally, U373MG cells were also transfected after 3 weeks preincubation with 1 mM lithium (Li), 75 µg/ml valproic acid or 10 ng/ml Haloperidol, respectively. Firefly and *Renilla* luciferase activities were determined sequentially with 20 µl of total cell lysates in 100 µl of luciferase assay reagent followed by addition of 100 µl of Stop & Glo reagent (Promega) per reaction. Chemiluminescence was measured for 10 sec in a liquid scintillation spectrophotometer (Berthold). *In vitro* methylated constructs were transfected in a ratio 5:1 (Fugene: DNA) using 0,4µg pGL3-

SLC12A6 constructs and 0,1µg pGL4.74 control vector. Luciferase expression was assayed under basal, dexamethasone and PMA stimulated conditions.

Experiments were done in triplicates and derived from 4 independent trials. Promoter activities are presented as Relative Light Units (RLUs) calculated as the quotient of the individual firefly luciferase driven value (promoter activity) divided by the correspondent *Renilla* luciferase driven value (internal control). Non parametric Mann – Whitney U-Test was used to calculate statistical differences between the groups investigated. Calculations were done both with and without statistical outliers, resulting in almost identical outcome. The results presented here are based on a sample where statistical outliers are included.

2.3.7 Pharmacological studies

EBV-transformed lymphocytes of heterozygous patient 834 (Meyer et al. 2005) were cultured in RPMI 1640, supplemented with 15 % FCS, 2 % L-glutamine, 1 % Pen/Strep and 0,2 % NEAA at 37°C in a humified atmosphere at 5 % CO_2. Cells were incubated with and without 1 mM Li, 75 µg/ml valproic acid or 10 ng/ml Haloperidol for 21 days. DNA was isolated and assayed for *SLC12A6* promoter methylation status as described above.

2.3.8 Bioinformatic analysis

Alternative promoters of *SLC12A6* were computationally analyzed using the BIMAS promoter scan software (BIMAS promoter scan; http://www-bimas.cit.nih.gov/molbio/proscan), and by comparison with recent predictive annotation of default epigenetic states and predicted DNA methylation of all human CpG islands (Bock et al. 2006; Bock et al. 2007). Predictions were obtained from

http://neighborhood.bioinf.mpi-inf.mpg.de/CpG_islands_revisited/. Transcription factor binding sites were analyzed using TRANSFAC 10.1®.

2.4 Results

Polymorphic promoter sites as well as transcription initiation sites of *SLC12A6* are shown in figure 2.1. Location of luciferase-constructs, restriction sites, and transcription factor binding sites revealed by using TRANSFAC 10.1 software are indicated.

2.4. Methylation

Genomic DNA was extracted from blood of patients carrying the rare promoter allele. Since a CpG site is created in the upstream G allele but not the A allele, we examined

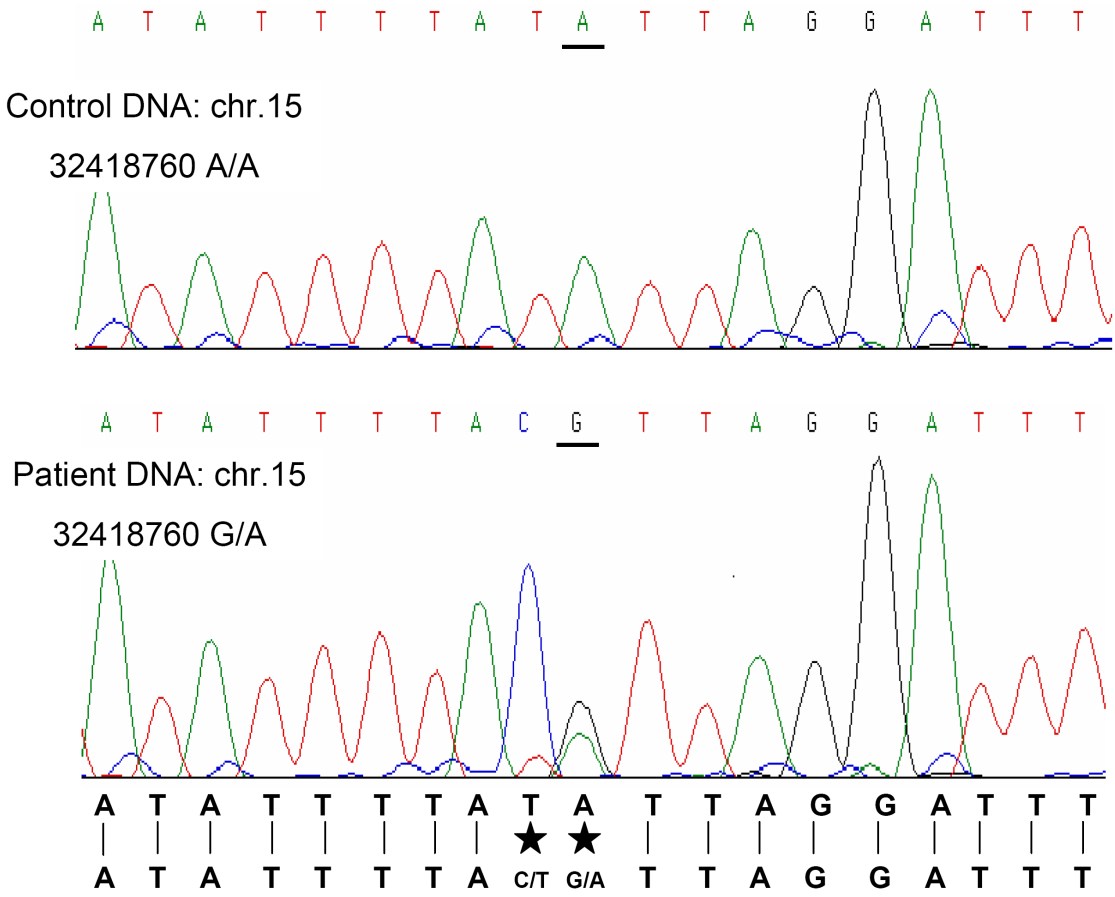

Figure 2.2: Electropherogram of *SLC12A6* promoter fragments

Electropherogram indicating directly sequenced PCR products of *SLC12A6* promoter fragments, of bisulfite-modified DNA deriving from individuals homozygous (A/A) and heterozygous (G/A) for the nt 32418760 SNP. The position of the upstream SNP is underlined; methylation of the adjacent C nucleotide is indicated by an asterisk**.**

the methylation status of the adjacent 5' cytosine. For this purpose, DNA samples were treated with bisulfite and subsequently sequenced as described above (for results, see figure 2.2). DNA of healthy and affected individuals heterozygous for the *SLC12A6* promoter variants were assayed for their DNA methylation pattern. The 5' cytosine of the upstream G variant was found methylated in all healthy controls (853 and 743) and affected family members (834, 744 and 999; Meyer et al. 2005).

Aim of this experiment was to investigate in principal whether the cytosine neighbouring the upstream SNP can be found in a methylated state. Therefore, quantification of DNA methylation (as described elsewhere; Moser et al. 2007) was not performed. For reasons of sample availability, DNA methylation analysis was performed on lymphocyte DNA. Since tissue-specific DNA methylation seems to be confined to no more than 5% of human promoters (Eckhardt et al. 2006; Song et al. 2005), we believe that it is plausible to extrapolate our findings from blood to brain tissue. To assess the functional effect of both the SNP and its DNA methylation status on gene regulation, we subsequently analyzed the effect of the different alleles on gene expression.

2.4.2 Luciferase Reporter Gene Assays

To investigate the impact of the polymorphic sites on gene regulation, the entire portion of the promoter as well as sub-fragments were fused to a luciferase reporter gene as described above. As shown in figure 2.3, the presence of G alleles (pGl3-G-G) reduced basal luciferase expression in comparison to the abundant A alleles (pGl3-A-A; $p<0.001$). Dexamethasone significantly stimulated luciferase expression in the presence of A (pGl3-A-A), but not G alleles (pGl3-G-G; $p<0.001$), thus pointing to selective glucocorticoid-mediated regulation of the abundant promoter variants even as there is no GRE motif present in the promoter sequence. PMA stimulates expression of both G

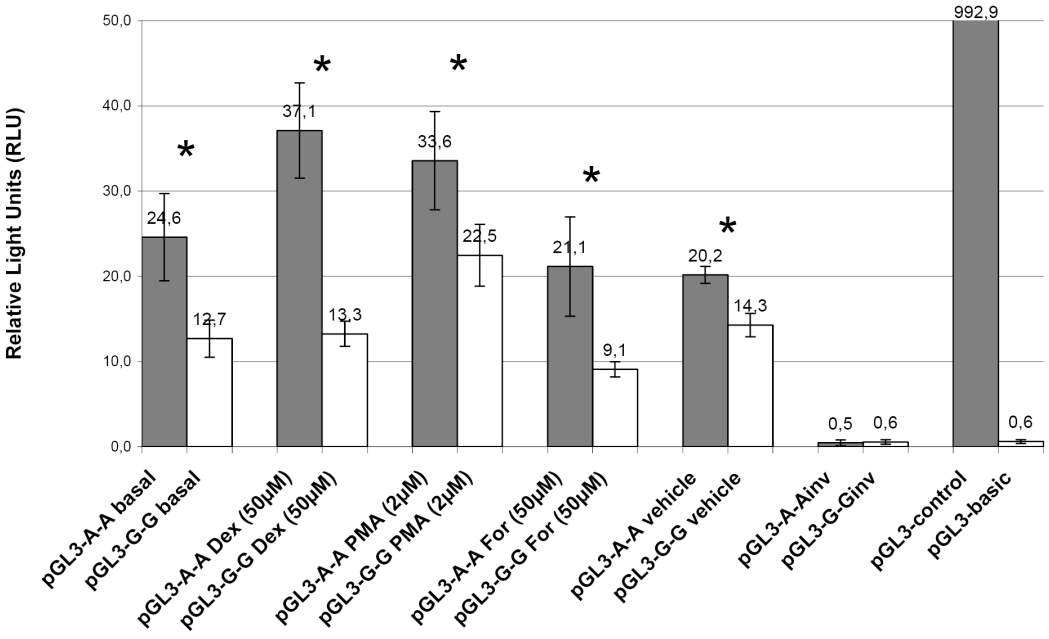

Figure 2.3: Basal and induced activity of the *SLC12A6* 5' regulatory region
Relative light units comparing pGL3-A-A vs. pGL3-G-G-specific *SLC12A6* promoter constructs. (*p<001)

and A alleles but the promoter activity of the A allele remained significantly higher (p<0.001). Therefore, protein kinase C-mediated regulation is likely but probably does not act directly at the polymorphic sites. As there are several activator protein 1 (Ap-1) binding elements present in the *SLC12A6* promoter, increased promoter activity may be induced by the PMA-induced PKC activation of these transcription factors.

Forskolin did not affect promoter activity, and the results were almost identical to unstimulated conditions for both *SLC12A6* promoter constructs. This provides evidence that PKA-mediated signal transduction pathways are not involved in the regulation of *SLC12A6* expression. Under forskolin stimulation, activity of the A allele is, as before, significantly higher compared to the G alleles (p<0.001). To distinguish the functionality of the polymorphic sites, the upstream polymorphic site was deleted as described. As shown in figure 2.4, deletion of the respective upstream site by *Stu*I digestion equalizes expression rates of both A- and G-specific promoter constructs

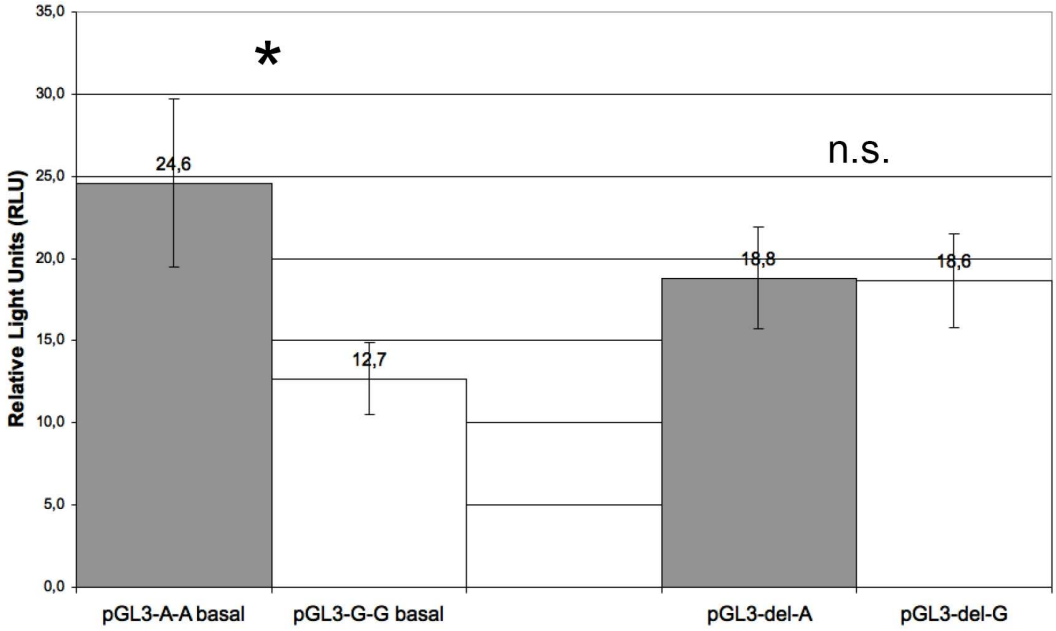

Figure 2.4: *SLC12A6* promoter activity in dependence of the upstream polymorphic site
SLC12A6 promoter activity before and after deletion of the upstream G/A polymorphic site (*p<001).

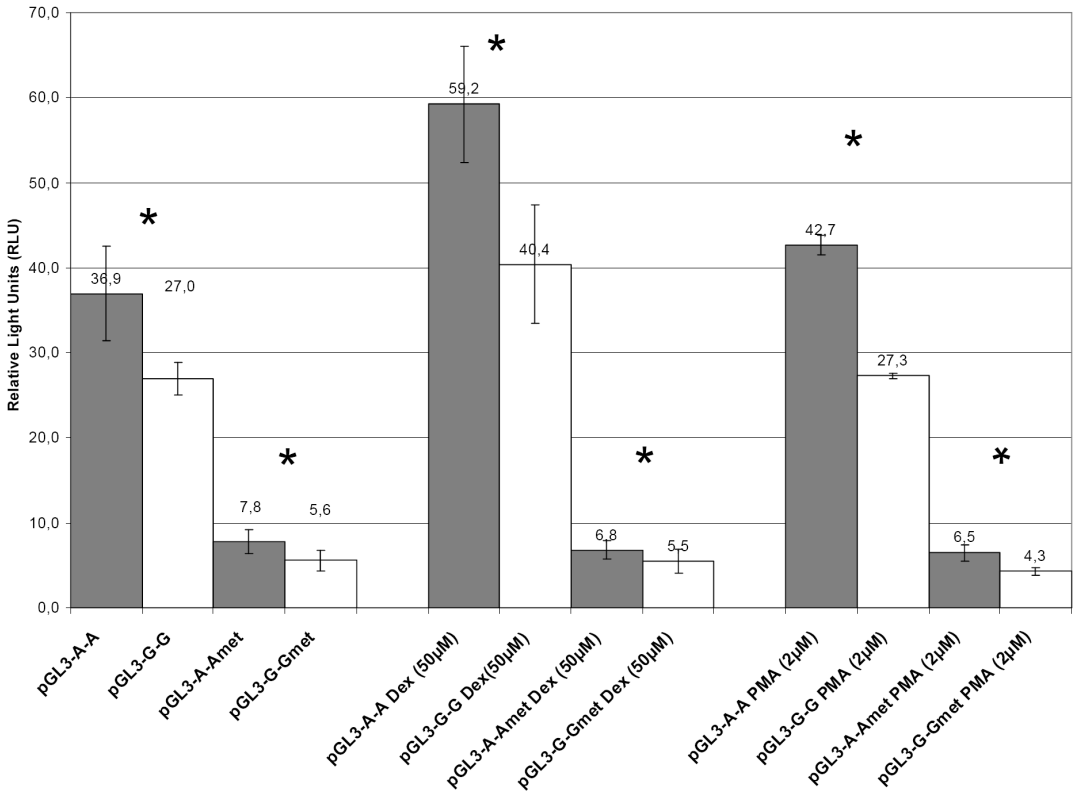

Figure 2.5: *SLC12A6* promoter activity before and after *in vitro* methylation
Basal and induced activity of the *SLC12A6* 5' regulatory region before (pGL3-A-A vs. pGL3-G-G) and after *in vitro* methylation (pGL3-A-Amet vs. pGL3-G-Gmet; all *p<002)

(pGl3-del-A; pGl3-del-G; *p*=.16) to a medium level, pointing to functional relevance exclusively of the 5' polymorphic site. Interestingly, the promoter activity was found to be decreased for the pGl3-del-A construct, whereas it was slightly increased in the vector carrying the G variant *SLC12A6* promoter fragment (pGl3-del-G). *In vitro* methylation of pGL3-A-A and pGL3-G-G constructs led to almost silenced promoter activity. After *in vitro* methylation, pGL3-G-G activity was significantly lower (all p<.02) compared to that of the pGL3-A-A (Figure 2.5) under all conditions assessed, may be due to the additional methylation site in the G-allele.

2.4.3 Effects of mood stabilizers and antipsychotic drugs on CpG methylation and SLC12A6 promoter activity

Since it has been proposed that *SLC12A6* resembles a positional candidate gene for bipolar disorder and subtypes of schizophrenia (Delpire and Mount 2002; Meyer et al. 2005), we investigated whether the *SLC12A6* polymorphic promoter sites are prone to stimulation by common mood stabilizers such as lithium and valproic acid, which are both commonly used in the treatment of bipolar disorder, and the antipsychotic drug haloperidol, frequently used in the treatment of psychoses. However, presence of haloperidol, valproic acid or lithium had no effect on reporter gene expression in our test system as they also had no influence on promoter methylation (data not shown).

2.4.3 Computional epigenetics

Genes with strong CpG island promoters are often ubiquituously expressed, showing robust transcription across a wide range of tissue types (Vinogradov 2005). In constrast,

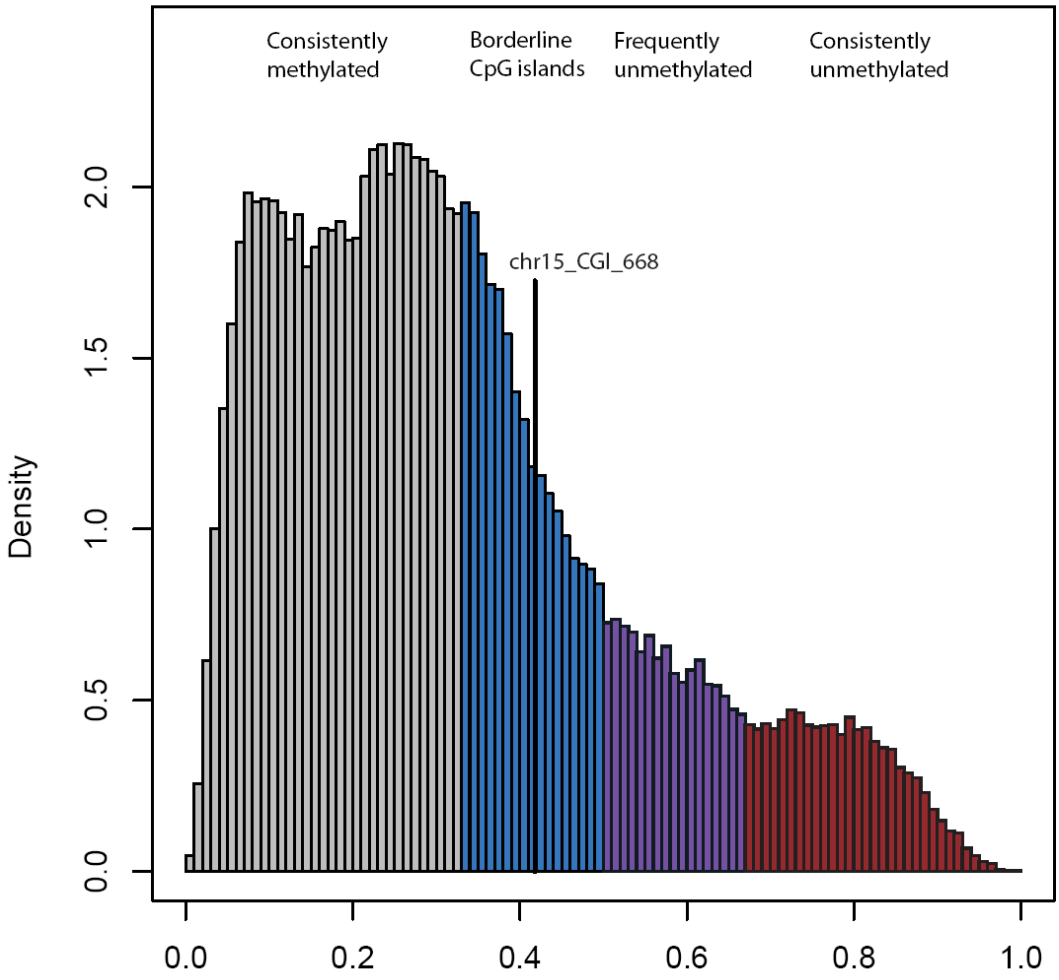

Figure 2.6: Predicted CpG Island Strength

Histogram of the predicted CpG island strengths for all CpG islands in the human genome. The x-axis gives the CpG island strength as predicted by the combined epigenetic score (Bock et al. 2007), and the y-axis gives a density estimate, normalized to a total area of one. From left to right, CpG islands are predicted to exhibit a more open and transcriptional permissive chromatin structure. The orthogonal line marks the promoter CpG island of *SLC12A6*.

genes with less CpG-rich promoters are more susceptible to regulation by variable methylation levels (Eckhardt et al. 2006). By comparison with a recently published prediction of the strength and expected epigenetic state of all CpG-rich regions in the human genome (Bock et al. 2007), we found that *SLC12A6* is regulated by a weak CpG island promoter (Figure 2.6). With a CpG island strength prediction of 0.418 on a scale between zero and one (highlighted by a vertical line in Figure 2.6), it may be sensitive to a single additional CpG upstream of the core promoter. A recent study of the

genomic determinants of DNA methylation variation (Bock et al. 2008) lends further support to a potential epigenetic function of the G/A polymorphism in the upstream region of *SLC12A6*. Using the methodology of this study, the genomic region between the SNP and the transcription start site was predicted to exhibit relatively large inter-individual variation but also consistent conservation of the DNA methylation status of specific CpGs (the region's predicted h_i score is 0.03). Furthermore, we note that a genomic polymorphism that becomes functional via its *cis* effect on epigenetic regulation has been reported previously by a study on Beckwith-Wiedemann syndrome (Murrell et al. 2004), in which loss of imprinting at the *IGF2* gene could be associated with four neighbouring SNPs. In conclusion, while a rigorous proof that the upstream G/A polymorphism is functional due to epigenetic rather than genetic regulatory effects is still outstanding, we have accumulated several lines of experimental and computational evidence that DNA methylation may contribute to its effect on gene expression.

2.5 Discussion

The potassium-chloride co-transporter 3 gene represents a strong positional and functional candidate for psychoses, including bipolar disorder and schizophrenia (Delpire and Mount 2002; Meyer et al. 2005; Uyanik et al. 2006). Here, we provide a functional mechanism for a rare promoter variant of *SLC12A6,* associated with bipolar disorder (Meyer et al. 2005). It could be demonstrated that the less frequent allele of the investigated SNP can lead to DNA methylation at a CpG site, and was associated with differences in the regulation of gene expression in our test system.

As shown by Escher and colleagues (Escher et al. 2005), plasmids can be methylated after transfection. Therefore we tested *SLC12A6* promoter activity comparing G vs. A-alleles. To ensure complete plasmid methylation, constructs were also assayed after in vitro methylation. Under all conditions tested the *SLC12A6* G promoter allele showed significantly lower promoter activity compared to the A-allele. Deletion of the upstream polymorphic region led to equal promoter activities of both constructs indicating that the *SLC12A6* upstream promoter SNP is functional and responsible for altered promoter activity. The SNP investigated here was found to be associated with bipolar disorder in a case control study (Meyer et al. 2005). Results of the present study now demonstrate functionality of the upstream SCL12A6 promoter SNP. We propose that its functionality might be modulated by DNA methylation, known to be influenced by developmental and environmental factors in a time and tissue dependent manner and therefore may contribute to the pathogenesis of (psychiatric) disorders.

SLC12A6 is involved in cell proliferation processes (Shen et al. 2004) and mutations of this gene cause a severe neurological disorder frequently associated with psychosis (Filteau et al. 1991; Howard et al. 2002a; Howard et al. 2003; Howard et al. 2002b). The involvement of this gene in psychopathology supports the developmental hypothesis of psychoses (Isohanni et al. 2001; Jones et al. 1994; Weinberger 1995)

which suggests that subtle malfunction of axon guidance processes in the developing brain may lead to cognitive and behavioral impairment. It is noteworthy that we are not studying novel mutations here but polymorphic variants, of which the G alleles are rare but nonetheless present in the gene pool in Hardy-Weinberg equilibrium with their respective pendants. According to the theory of complex disorder inheritance (Cannon 2005), functional variants of this kind may act together with others in the pathogenesis of certain complex diseases and personality traits (Kohn 2005). In addition, it has been proposed (Liu et al. 2005) that rare variants may be of importance to the understanding of pathogenesis of complex inherited disorders.

Our findings support observations made in *Slc12a6* knockout studies revealing a gene dosage effect in *Slc12a6* null compared to heterozygous knockout mice (Boettger et al. 2003; Howard et al. 2003; Howard et al. 2002b; Jentsch 2005; Rust et al. 2006). In humans, *SLC12A6* deficiency leads to Andermann`s syndrome. Interestingly, individuals possessing only one functional *SLC12A6* allele do not show any clinical or even subclinical phenotype. However, here we provide evidence for a gene variant leading to a DNA methylation site with functional consequences on *SLC12A6* promoter activity. Such methylation-sensitive variants may represent targets for epigenetic modification induced by environmental factors (Weaver et al. 2004), and become functionally relevant only after the occurrence of disease precipitating factors such as chronic stress, critical life events or pathogen exposure (Fraga et al. 2005; Weaver et al. 2004; Weaver et al. 2005).

We speculate that the newly identified DNA methylation site may represent a polymorphism with a *cis*-regulatory, epigenetic mode of functioning. Since the role of environmental factors in influencing the DNA methylation and other epigenetic modifications is now well-established (Peaston and Whitelaw 2006), it seems likely that the presence of a *SLC12A6* promoter variant, by introducing an additional DNA

methylation site, might mediate a functional connection between environmental factors such as psychosocial stress and the increased susceptibility to psychopathology conferred by a genetic variant (de Kloet et al. 2005). The possible impact of environmental factors on the epigenotype has recently been demonstrated. For instance, it could be shown that natural variations in maternal care (Weaver et al. 2004), nutrition (Weaver et al. 2005; Wolff et al. 1998) and drug treatment (Alonso-Aperte et al. 1999; Shimabukuro et al. 2006; Weaver et al. 2004) have an impact on promoter methylation in rats. Interestingly, the epigenotype shows differences even in human monozygotic twins over the life span, reflecting the influence of lifestyle and ageing on the epigenotype (Fraga et al. 2005).

Promoter activities were found to be decreased after deletion of a 943bp *Stu*I fragment for the pGl3-del-A construct, whereas it slightly increased in the vector carrying the G variant *SLC12A6* promoter fragment (pGl3-del-G). This is supporting our hypotheses that CpG methylation adjacent to the upstream G/A polymorphic site could lead to a slightly more condensed DNA organization in the G-allele, whereas the DNA organization in the A-allele lies open for transcription. After deletion of the upstream polymorphic region, elements available for epigenetic modulation silencing promoter activity in the G-allele/ enhancing promoter activity in the A-allele, respectively, were excised leading to almost identical promoter activities of the constructs investigated.

It has to be mentioned as a limitation that, as we did not have access to brain tissue of the appropriate genotype, DNA methylation pattern in carriers of the G allele was investigated in peripheral tissue. Given the high tissue specificity in gene expression patterns, brain tissue might have a different DNA methylation pattern. However, we could demonstrate in principle that the *SLC12A6* upstream G /A SNP can lead to allele specific methylation and that presence of the G allele is associated with reduced promoter activity– possibly brought about by DNA methylation.

In silico analysis using TRANSFAC 10.1® did not reveal altered transcription factor binding sites comparing A and G-allele. It cannot be ruled out that unknown *cis*- or *trans*-acting factors may affect allele specific promoter activity. As the upstream palindromic sequence TTAC**A**TT is abolished in the presence of the rare G nucleotide (TTAC**G**TT) putative transcription factor binding efficiency could be altered and also be fine tuned by cytosine methylation in a time, tissue and environment dependent manner.

On the other hand, it was hypothesized that methylated DNA can facilitate histone deacetylation by recruiting chromatin remodelling enzymes such as histone deacetylases (HDACs) via the action of methyl-CpG binding domain proteins (MBDs) like MeCP2 (Tsankova et al. 2007). In the *SLC12A6* G-allele, a palindromic sequence of sense and antisense strand is created (5'-AC**G**T-3'), possibly representing a MBD binding site. This putative protein binding site furthermore could be modulated by cytosine methylation leading to enhanced MBD binding and decreased promoter activity. Both possibilities, higher binding efficiency for a positive regulator of gene transcription in the A-allele or higher binding efficiency of a negative regulator in the G-allele may help explaining the putatively causative effect of this variant in family 11 (Meyer et al. 2005).

Future studies are warranted to identify putative transcription factors selectively binding in the *SLC12A6* upstream polymorphic region. It will need to be clarified whether CpG methylation underlies functionality at this site or if transcription factor binding is altered due to the presence of the G allele per se. Even if the reduction in gene expression is independent of CpG methylation, this study provides data for the functionality of the investigated SNP.

Furthermore, as DNA methylation and histone deacetylation act in a synergistic fashion in order to regulate gene transcription (Attwood et al. 2002), *SLC12A6* SNP-specific

chromatin immuno precipitation assays (ChiP-assays) to identify histone acetylation pattern influencing *SCL12A6* DNA organization are warranted. Additionally, it is conceivable that alternative *SLC12A6* first exon usage, as already described for the neuronal nitric oxide synthase (nNOS, Reif et al. 2006; Saur et al. 2004), can be selectively modulated by promoter SNPs. Therefore, tissue-specific expressions of the different *SLC12A6* transcripts have to be elucidated in further studies.

In conclusion, the present study provides evidence that the upstream *SLC12A6* G/A promoter SNP is functional not only by changing the DNA primary structure but also by influencing the allelic epigenotype and consequently by influencing the chromatin organization. The upstream *SLC12A6* "G/A" SNP could represent a functional link between genetic variation and epigenetic modification.

CHAPTER 3

The glucocorticoid receptor gene exon 1-F promoter is not methylated at the NGFI-A binding site in human hippocampus

Dirk Moser, Anne Molitor, Robert Kumsta, Thomas Tatschner, Peter Riederer &

Jobst Meyer

3.1 Abstract

Recent research has demonstrated that early life experience, such as variation in maternal care, can have a profound impact on the physiological and endocrine stress response of *Rattus norvegicus*. Low maternal care resulted in increased methylation of the nerve growth factor-inducible protein A (NGFI-A, EGR1) binding site located in the hippocampal glucocorticoid receptor gene (*Nr3c1*) exon 1_7 promoter, leading to decreased *Nr3c1* expression, which results in a reduced efficiency of glucocorticoid-mediated negative feedback on hypothalamus-pituitary-adrenal axis activity. The human glucocorticoid receptor gene (*NR3C1*) has a highly similar 5' structure compared to the rat, and the human alternative exon 1-F is the orthologue to the rat exon 1_7. Based upon the evidence from rats, and the high sequence identity of the regulatory sequences, we examined the methylation pattern of the corresponding NGFI-A binding site in the human glucocorticoid receptor exon 1-F specific promoter in post mortem hippocampal tissue.

In contrast to the findings in rats, neither of the two CpG motifs within the NGFI-A binding site was methylated in the 32 subjects investigated. These observations might reflect different promoter methylation patterns in humans and rats.

3.2 Introduction

Mental disorders constitute a major burden for society. Across 28 European countries with a total population of 466 million, 127 million people, or 27 % are affected by mental or neurological disorders. These disorders figure among the leading causes of disease and disability (Fact sheet Europe 03/03: Mental health in the WHO European Region), and are associated with immense total costs of over 290 billion Euros per year (Wittchen and Jacobi 2005). The relationship of mental disorders to stressful life events is well established, both in epidemiological and clinical samples (Paykel 2003). The stress response has evolved as a highly adaptive reaction that ensures survival when an organism is confronted with physiological or psychological challenge. One important stress response system is the hypothalamus-pituitary-adrenal (HPA) axis, which mediates the endocrine stress response. HPA axis activity is controlled by negative feedback mechanisms through activation of the glucocorticoid receptor (NR3C1, GR;(de Kloet et al. 2005; Jacobson and Sapolsky 1991). Reports from both animal (Howell and Muglia 2006) and human studies (Holsboer 2000; Neigh and Nemeroff 2006; Pariante and Miller 2001) have associated impaired NR3C1 functioning in the etiology of depression or depression-like behaviour, and altered HPA axis regulation has been associated with many psychiatric and psychosomatic disorders (Young 2004). Although the relationship of mental disorders to stressful life events is well established, the question remains why some people who are exposed to chronic stress develop mental disorders while others do not.

To answer this question, it is becoming increasingly important to identify psychobiological mechanisms (Singer 2001), linking environmental influences to changes at the molecular level that predispose to, or sustain disease processes.

In a rodent model, research has emerged linking early environmental influence, i.e. variation in maternal care, to behavioral and neuroendocrine alteration in adult life (Meaney 2001; Weaver et al. 2004). The offspring of rat mothers showing high maternal care were less fearful and showed reduced hypothalamus-pituitary-adrenal (HPA) axis response to stress. On the molecular level, the reduced HPA axis responsiveness in offspring of high caring mothers was associated with increased hippocampal Nr3c1 mRNA expression and enhanced glucocorticoid (GC) feedback sensitivity. Cross-fostering studies revealed that most of these effects are non-genomically transmitted from one generation to the other, suggesting epigenetic mechanisms (Francis et al. 1999). Weaver and associates (2004) demonstrated that variation in maternal care led to stable alteration of DNA methylation pattern. Low maternal care resulted in increased methylation of the early growth response protein 1, alternatively termed nerve growth factor-inducible protein A (EGR1, NGFI-A) binding site located in the *Nr3c1* gene exon 1_7 promoter, leading to decreased *Nr3c1* expression. Expression of exon 1_7 mRNA seems to be unique to hippocampus, so that methylation of exon 1_7 specifically reduces *Nr3c1* expression in hippocampal sites (Weaver et al. 2004).

Given this evidence from animal studies, we examined the corresponding site of the human orthologous gene (*NR3C1*) promoter in 32 post mortem hippocampus tissues with respect to their methylation pattern. As an internal control, we co-examined the methylation pattern of the Insulin-Like Growth Factor II gene (*IGF2*), a gene that is imprinted on the maternally inherited allele.

The aim of the present study was to clarify whether similar epigenetic processes exist for the human glucocorticoid receptor gene promoter as observed in the rat.

3.3 Methods:

DNA was isolated from human hippocampus *post mortem* tissue (Gsell et al. 1993) of 32 subjects (12 males / 20 females, mean age 80,1 ± 9,0 years / 82,6 ± 6,3 years with various diagnoses: Parkinson's disease (9), presenile dementia – Alzheimer's type (2), senile dementia – Alzheimer's type (SDAT; 8), dementia (8), and 5 without any diagnoses) using QIAmp DNA Micro Kit (Qiagen; Hilden).

3.3.1 Sodium bisulfite mapping / methylation specific PCR (MSP):

The human *NR3C1* exon 1-F promoter region (104 bp around the NGFI-A binding site) corresponding to the rat exon 1_7 regulatory genomic region was investigated (GenBank accession numbers: AJ877168, AJ271870). One µg sodium bisulfite-treated (Frommer et al. 1992; Grunau et al. 2001) hippocampal, genomic DNA was subjected to nested PCR amplification using flanking primers (forward: -142763905- 5`-GTTGTTATT**XG**TAGGGGTATTGG -3`- 142763883; reverse: 142763770- 5`-AAACCAC**CX**AATTTCTCCAA - 3`-142763789; sequence numbering is with respect to the nucleotide position on human chromosome 5, according to the sequence published by the International Human Genome Sequencing Consortium; UCSC Genome Browser Golden Path of May 2004). The thermocycler protocol involved an initial denaturation cycle (5 min, 94 °C), 34 cycles of denaturation (30 s, 95 °C), annealing (1 min, 56 °C) and extension (30 s, 72 °C), followed by a final extension cycle (7 min, 72 °C) terminating at 4 °C. The PCR product (136 bp) was used as a template for subsequent PCR reactions using nested primers (forward: 142763876 -5`- TTGT**XG**TTAAGGGGTAGA-3`- 142763859; reverse: 142763773 – 5`-CCAC**CX**AATTTCTCCAATTTC -3`- 142763793). After gel

purification, the nested PCR products (104 bp; including 9 CpG sites) of all 32 subjects were directly sequenced for qualitative methylation analysis.

For methylation quantification, the PCR products of ten subjects (4 Parkinson's disease; 5 SDAT; 1 control) were sub-cloned (Original TA cloning kit, Invitrogen), and amplified in bacteria. For each subject, ten plasmids containing the cloned *NR3C1* exon 1-F promoter DNA fragment were sequenced (Big Dye Terminator Kit, Perkin Elmer) using an ABI 310 genetic analyzer.

In order to control for differential methylation pattern in our post mortem tissue, a 255 bp fragment of the naturally imprinted *IGF2* gene (Ak025719; chromosome 11: pos. 2111299 - 2111553;forward: 5`-GGGAAAGGGGTTTAGGATTTTTAT-3`; reverse:- 5`-ATAATTTACTCCCCCTTCAACCTC -3`;(Dupont et al. 2004) was PCR amplified after bisulfite modification. DNA for control experiments derived from five randomly chosen subjects (2 Parkinson's disease; 2 SDAT; 1 control) out of the ten examined for their *NR3C1* methylation status. The thermocycler protocol involved an initial denaturation cycle (5 min, 94 °C), 50 cycles of denaturation (30 s, 94 °C), annealing (45 s, 60 °C) and extension (30 s, 72 °C), followed by a final extension cycle (7 min, 72 °C) terminating at 4 °C. All PCR`s were performed using 10 pmol of each primer, 200 µM dNTP, 1-2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 2.5 U Taq Polymerase (Gibco BRL) in a total volume of 50 µl. The methylation quantification was done as described for the *NR3C1* gene fragments.

Quantification is given in percent according to the methylated cytosines identified out of ten CpG dinucleotides investigated for each position.

3.4 Results

As shown in Figure 3.1, a 5'-GCGGGGGCG-3' motif, encompassing two CpG sites (CpG^{6+7}), is present in the human *NR3C1* exon 1-F promoter. This sequence represents the consensus binding site for the transcription factor NGFI-A as observed for the rat exon 1_7

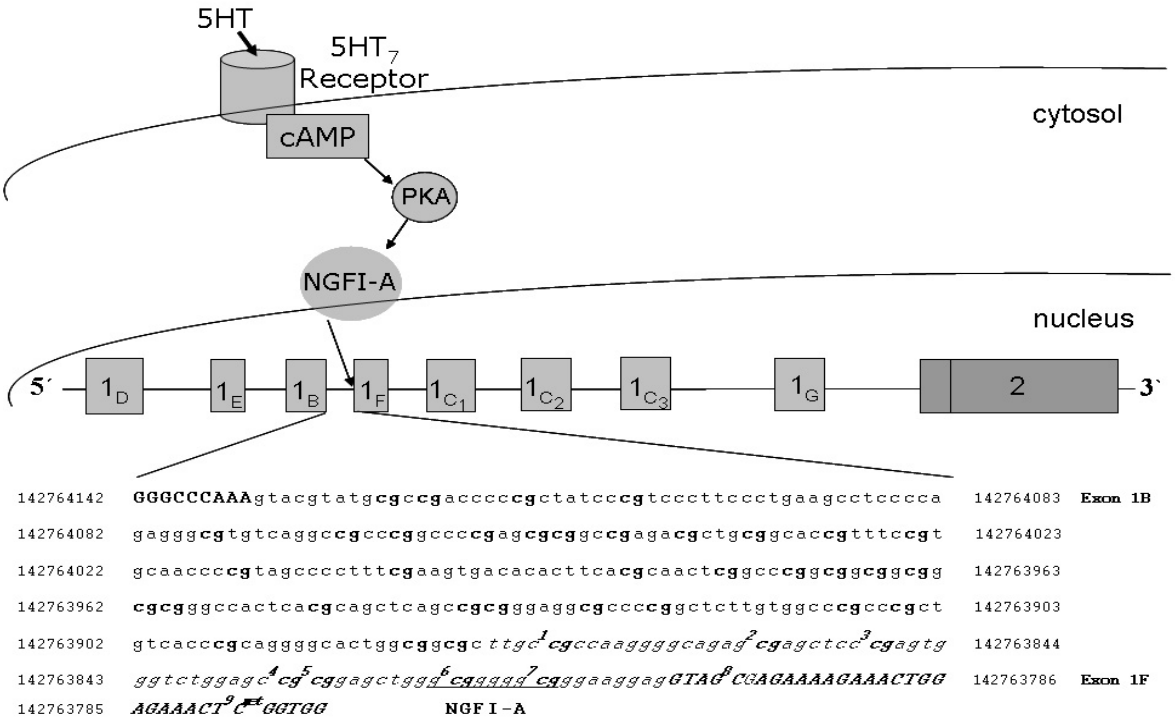

Figure 3.1: Model for *NR3C1* exon 1-F specific activation in hippocampus tissue.
Numbering is with respect to human chromosome 5. Small letters indicate the *NR3C1* 1-F regulatory region. The NGFI-A binding site is underlined. Capital letters symbolizing exonic sequences. CpG dinucleotides are shown in bold letters, italic letters indicate the sequence investigated; CpG`s investigated in this study are numbered in 5`- 3`direction.

(Dupont et al. 2004; Turner and Muller 2005; Weaver et al. 2004). Multiple sequence alignment revealed that this sequence is highly conserved among different species (Fig. 3.2), pointing to a functional relevance of the motif.

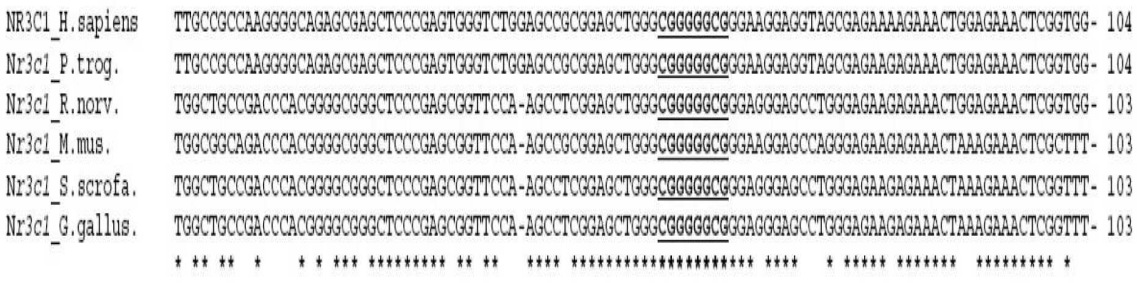

Figure 3.2: Alignment of the 104 bp *NR3C1* 1-F-specific regulatory region for 6 species
Alignment was performed using clustalW software; http://www.ebi.ac.uk/clustalw/). The NGFI-A binding site is underlined. Asterisks indicate 100 % identity. (Accession numbers: Homo sapiens - NM_000176; Pan troglodytes - NM_001020825; Rattus norvegicus - NM_012576; Mus musculus - NM_008173; Sus scrofa - NM_001008481; Gallus gallus- NM_001037826)

3.4.1 Methylation

In contrast to the methylation pattern described for the rat (Weaver et al. 2004), none of the two CpG motifs (CpG $^{6+7}$; Fig. 3.3) within the NGFI-A binding site of the *NR3C1* exon 1-F promoter isolated from human hippocampal tissue was found methylated in the 32 subjects investigated in this study. Furthermore, none of the remaining CpG sites (CpG $^{2-8}$; Fig. 3.3) present in the promoter fragment investigated, and corresponding to the orthologous CpG sites 12 – 17 of the rat exon 1_7 (Weaver et al. 2004) were found to be methylated in humans. In contrast, all 15 CpG sites of the corresponding rat exon 1_7 promoter are differentially methylated; the extend being highly dependant on the intensity of maternal

care (Weaver et al. 2004). Besides, we found solely two CpG sites being methylated. CpG^{1} (67 basepairs downstream of the *NR3C1* exon 1-F transcription start side) was unmethylated in 9 subjects, and methylated to 10 % in a single subject (Figure 3.3), whereas CpG^{9}, located in exon 1-F, was methylated ranging from 80 - 100 % in all subjects.

For the controls, we found methylation levels of the naturally imprinted *IGF2* being inter-individually highly variable (Figure 3.4) but comparable to the data published for lymphocytes (Dupont et al. 2004; Heijmans et al. 2007), confirming that neither age, dementia or Parkinson's disease did interfere with cellular methylation in the sample investigated.

3.5 Discussion

Epigenetic processes constitute fundamental mechanisms in development as they ensure cell differentiation and body compartmentalization by differentially silencing or activating genes. Recently, Weaver and colleagues (Weaver et al. 2004) provided evidence that cytosine methylation, one of the key mechanisms of cellular programming, could be considerably influenced by environmental factors such as the intensity of maternal care in the rat model. Not surprisingly, the study of Weaver and associates was welcomed as one of the first publications, which points to cellular mechanisms constituting a functional link between behavioural variation and gene regulation (Dolinoy et al. 2006; Rakyan and Beck 2006; Weaver et al. 2004; Weaver et al. 2005; Weaver et al. 2006). Weaver and co-workers reported on methylation pattern of the exon 1_7 specific *NR3C1* promoter in general, and of two CpG dinucleotides in the NGFI-A binding site in particular, where the 5' CpG displayed differences in methylation dependent on maternal care, whereas the 3' CpG site was constantly methylated, independent of maternal care.

The ability of this cellular system to serve as an adaptive, non-genetic programming process, capable of ensuring the juvenile, immature or adult individual's coping with favourable or adverse environments, has since been discussed. In this study, we show that the mechanism presented by Weaver and associates is probably different between humans and rat, despite the fact that the sequence investigated here is highly conserved among different species (Fig. 3.2). We found CpG^{1}, located in the promoter, poorly methylated (10 % in 1 patient), and the adjacent investigated $CpG's^{2-8}$ unmethylated in all subjects. The CpG^{9}, located in *NR3C1* exon 1 F, was found highly methylated in all subjects investigated (Fig. 3.3). The high exonic methylation observed here is concordant with the literature,as

coding regions downstream of promoters usually contain CpG dinucleotides that are frequently methylated (Attwood et al. 2002). The almost unmethylated human *NR3C1* 1-F promoter points towards differences in glucocorticoid receptor gene methylation between humans and rats (Weaver et al. 2004; Weaver et al. 2005; Weaver et al. 2001).

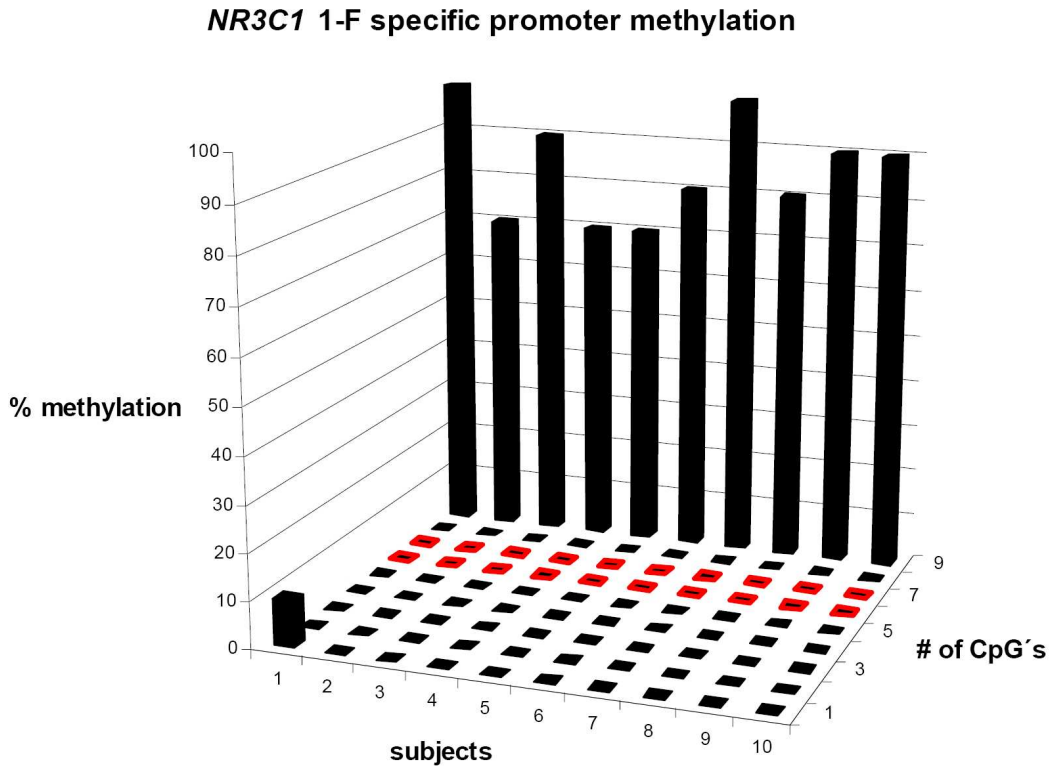

Figure 3.3: *NR3C1* exon 1-F specific promoter methylation
Percent methylation of nine CG dinucleotides investigated for the 104 bp *NR3C1* 1-F specific promoter fragment in 10 subjects. Red squares (CG $^{6+7}$) are representing the NGFI-A binding site.

In the rat, differential methylation in the NGFI-A binding site was observed at the 5' CpG only, whereas the 3` CpG was constantly methylated independent of maternal care. In our sample, neither the orthologue 5' CpG, nor the 3' CpG site of the NGFI-A binding site were found to be methylated in human hippocampus. Since no information on early environment was available for our subjects, the question whether environmental factors influenced methylation pattern cannot not be answered by this study. However, since differences between humans and rats were revealed also for the CpG with methylation status independent of maternal care, we hypothesize that basic *NR3C1* regulation may underlie different mechanisms in humans compared to the rat. The observation that none of the CpG´s was methylated more likely reflects a unitary process leading to an almost unique epigenotype as observed for the *NR3C1* 1-F 5' regulatory region in our sample.

Findings on epigenetic regulation processes are controversial. Fraga and colleagues (Fraga et al. 2005) showed that the methylation status of several genes is differentially altered in monozygotic twins over their life span. They pointed out that environmental factors exist in critical time periods of human development, which may even influence the individual's epigenotype in monozygotic twins. Given this evidence, differences in the *NR3C1* epigenotype of 32 unrelated subjects investigated here could be expected.

On the other hand, Eckhardt et al. (Eckhardt et al. 2006) did not find substantial inter-individual differences for DNA methylation by investigating 3 chromosomes with about 1.9 million CpG methylation sites. Furthermore, no effects of age were observed. In contrast to studies performed in humans and rodents (Fraga et al. 2005; Weaver et al. 2004), which demonstrated the generation of different epialleles during lifetime, our data

suggest that the human *NR3C1* 1-F promoter DNA methylation is ontogenetically more stable than expected, leading to an almost unmethylated promoter in older human beings. Additionally, not only variation in maternal care (Weaver et al 2004) but also nutritional methyl-donating substances such as methionine, choline, vitamin B12, and folic acid were shown to influence the methylation status and expression of several genes including the glucocorticoid receptor gene (Waterland et al. 2006; Weaver et al. 2004; Weaver et al. 2005). Furthermore, different pharmaceuticals such as valproic acid, haloperidol or trichostatin A (TSA) have an effect on DNA methylation (Alonso-Aperte et al. 1999; Chen et al. 2002; Manev and Uz 2002; Shimabukuro et al. 2006).

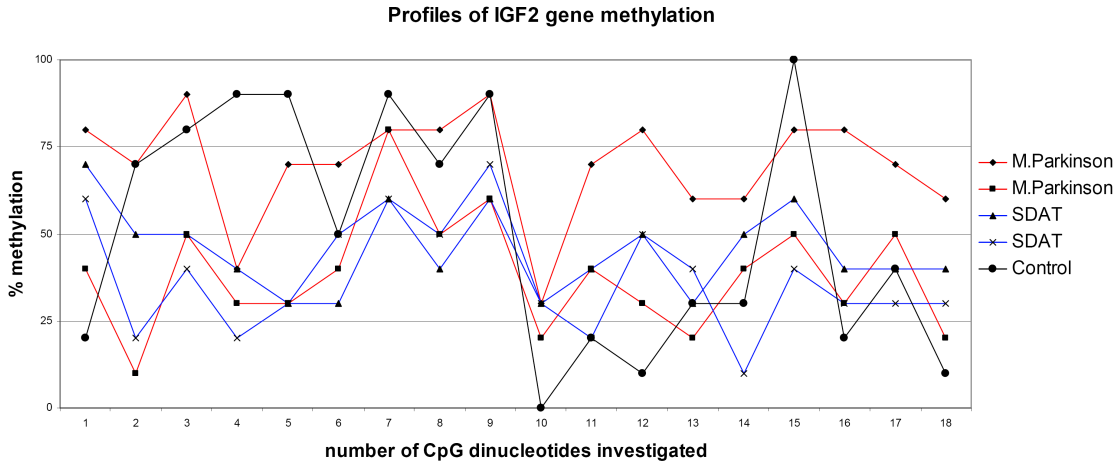

Figure 3.4: Profiles of *IGF2* DMR CpG methylation obtained by MSP
Analysis was performed using human post mortem hippocampus tissue (n = 5). Ten clones per sample were sequenced for analysis

By analyzing methylation pattern of *IGF2,* we could confirm that neither age, nor dementia or Parkinson's disease led to substantial demethylation. DNA demethylation has been documented in aging salmon, mice, rats, cows and humans, and occurs in various tissues including the brain (Richardson 2002; Wilson et al. 1987). Levels of *Igf2* expression decrease concomitantly with the degree and pattern of methylation in aged rat brain (Kitraki et al. 1993). Since we found that the *IGF2* methylation pattern in aged human brain is highly variable, we assume that the de-methylated *NR3C1* 1-F promoter is not based on a general age-dependent demethylation process.

However, we cannot rule out that epigenetic programming processes similar to that demonstrated by Weaver et al. (Weaver et al. 2004) may act at different sites of the human genome in different brain regions, which were not investigated in this study. For instance, Weaver et al. (2006) investigated 31,099 hippocampal transcripts with respect to different early life experiences. They could show that at least 3% of the whole mRNA was differentially expressed depending on intensity of maternal care, TSA treatment, or methionine application. These results indicate that there is a multitude of genes implicated in epigenetic programming processes.

The present study was performed in order to investigate whether similar basic epialleles, as observed in the rat, also exist in human hippocampus DNA. Certainly, our investigation is limited by the availability of human post mortem hippocampus tissue and especially by the lack of data on early childhood, critical life events, medication, etc. Therefore, the questions whether environmental factors modified the epigenome cannot be answered conclusively. However, we believe that it is unlikely that all subjects experienced the same environmental conditions resulting in the highly similar epigenotype as observed here.

Therefore, it can be hypothesized that *NR3C1* 1-F promoter in aged humans is constitutively unmethylated.

No data exist for glucocorticoid receptor epigenotype for hippocampi deriving from aged rats. Therefore, we cannot rule out that a similar methylation pattern might also be observed in aged rats independent of their early life experiences. In addition, we cannot exclude different methylation pattern in earlier life periods that were mediated by the environment or individual habits and were lost during aging. Therefore, investigation of hippocampal tissue deriving from younger donors is warranted.

To conclude, the human *NR3C1* 1-F specific methylation pattern seems to be subject to different mechanisms compared to the rat, albeit similar programming effects in general acting via variations in maternal care - as observed in the rat - cannot be ruled out for humans.

Chapter 4

Characterization of a glucocorticoid receptor gene (*GR, NR3C1*) promoter polymorphism reveals functionality and extends a haplotype with putative clinical relevance

Dirk Moser*, Robert Kumsta*, Fabian Streit, Jan Willem Koper, Jobst Meyer & Stefan Wüst

*these authors contributed equally

4.1 Abstract

Hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis has been associated with the etiology of major depression. One of the factors underlying altered glucocorticoid signalling might be variability of the glucocorticoid receptor gene (*GR*, *NR3C1*). *GR* polymorphisms have been associated with variability in glucocorticoid sensitivity and endocrine responses to psychosocial stress. Furthermore, a common *GR* SNP (rs10482605), located in the promoter region, has been associated with major depression. We performed functional characterization of this SNP *in vitro* using a reporter gene assay under different stimulation conditions. Furthermore, we genotyped 219 subjects previously genotyped for four common *GR* SNPs to further characterize *GR* haplotype structure.

The minor C allele of the rs10482605 SNP showed reduced transcriptional activity under unstimulated conditions and under different stimulation conditions in two brain derived cell lines. Linkage analyses revealed that the rs10482605 SNP is in high linkage disequilibrium with a A/G SNP in exon 9beta (rs6198), associated with relative glucocorticoid resistance and increased GRbeta mRNA stability.

We provide evidence that two functional *GR* SNPs in linkage disequilibrium are responsible for both regulation of *GR* expression and mRNA stability. This newly characterized haplotype could increase the risk for the development of stress related disorders, including major depression.

4.2 Introduction

There is a well established link between exposure to psychosocial stress and the development of psychopathology (Young 2004). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, one of the organism's major stress response systems, has been implicated in the onset and course of psychosomatic and psychiatric disorders, including major depressive disorder (Holsboer 2000). Numerous findings document HPA axis hyperactivity and altered responses in neuroendocrine challenge tests (dexamethasone suppression test or combined CRH/dexamethasone test; Holsboer 2001; Nemeroff 1996; Pariante and Miller 2001), supporting the hypothesis that alterations in glucocorticoid (GC) signalling might underlie an increased vulnerability for the development of major depression. The presumed central role of GR functioning in the development of psychopathology is supported by the observed correlation of HPA axis abnormalities with changes in GR function in major depression (Pariante 2004). Furthermore, reduced GR mRNA levels in the hippocampus and the frontal cortex of patients suffering from mood disorders and schizophrenia have been observed (Perlman et al., 2004; Webster et al., 2002). Consistently, several murine models with specific GR knockouts indicated that a primary defect in central GR signalling results in altered HPA axis regulation and changes in emotionally relevant behaviour (reviewed in Howell and Muglia 2006).

The significant heritability of major depression (Owen and Cardno 1999) and HPA axis activity (Bartels et al., 2003; Federenko et al., 2004; Wüst et al., 2004), the observation that healthy first-degree relatives of depressed patients show subtle changes of HPA axis function (Modell et al. 1998), and the fact that polymorphism in corticosteroid receptor genes can modulate the physiological stress response (Derijk et al., 2006; Kumsta et al., 2007; Wüst et al., 2004) have stimulated research linking genetic variation in HPA axis related genes with major depressive disorder. For instance, Binder et al. (2004) reported an association between a polymorphism in

the GR-regulating cochaperone FKBP5 and increased responses to antidepressant treatment. A growing number of studies have associated common GR gene SNPs with changes in metabolic parameters or variability in glucocorticoid sensitivity (Wust et al. 2004) and ACTH as well as cortisol responses to psychosocial stress. For instance, male homozygous carriers of the *Bcl*I G allele (rs41423247) showed decreased HPA axis responses (Kumsta et al., 2007; Wüst et al., 2004) to the Trier Social Stress Test (TSST) while the AG genotype of the rs6198 A/G SNP located in exon 9beta (henceforth referred to as 9beta A/G) was associated with enhanced ACTH and cortisol stress responses (Kumsta et al. 2007). In addition, two studies on *GR* polymorphisms have revealed an association with major depression. Carriers of the *Bcl*I GG genotype were at higher risk of developing a major depressive episode and carriers of the less frequent allele of the ER22/23EK polymorphism (located in exon 2; rs6189, 6190) showed faster clinical response to antidepressant therapy (van Rossum et al. 2006). In a Swedish sample, the association between the ER22/23EK SNPs with depression was replicated (van West et al. 2006) and furthermore, an association with major depression was observed for a SNP (rs10482605; NR3C1-1) in the promoter region of the *GR* in a Belgian sample (van West et al. 2006). The rs10482605 SNP is a T/C polymorphism, located in the promoter region of alternative exon 1C. The frequency of the minor C allele is 0.12 (dbSNP Build 127; http://www.ncbi.nlm.nih.gov).

This promoter polymorphism has not been functionally characterized so far. Given the association with major depression, and considering the location of the rs10482605 SNP in the gene's regulatory region, we sought to examine the transcriptional efficiency of the variant promoter by performing luciferase reporter gene assays under different signalling conditions in order to characterize the potential functionality of the different alleles in *GR* regulation. Furthermore, 219 subjects were genotyped for the rs10482605 SNP in order to analyze linkage disequilibrium to four previously genotyped common GR gene variants. Lastly, we aimed to

reanalyze the previously reported associations between *GR* haplotypes and endocrine responses to psychosocial stress as well as dexamethasone administration (Kumsta et al. 2007) now including the rs10482605 SNP in the analyses.

4.3 Materials and Methods

4.3.1 Genotyping

Initially, 601 subjects were genotyped for the *GR* SNPs ER22/23EK A/G (rs6189, 6190), N363S A/G (rs6195), *Bcl*I C/G (rs41423247) and 9beta A/G (rs6198). Subsequently, 219 subjects were selected for phenotyping according to their genotype. The stratification of the sample was performed in order to obtain roughly equally sized genotype groups and to allow an investigation of a sufficient number of the less frequent genotypes (Kumsta et al. 2007). For the present investigation, these 219 subjects were additionally genotyped for the rs10482605 SNP (van West et al. 2006). Genotyping was performed using standard PCR method. Primers used for amplification of a 317 bp RFLP specific PCR product are shown in Table 4.1. For allelic discrimination, the amplified PCR products were subsequently digested with the restriction enzyme *Bsl*I.

Primer used for genotyping: 5` → 3`
fw: GAGCTCCCGAGTGGGTCT
rev: AACCTGTTGGTGACGCTTG
Primer with restriction enzyme recognition sites (underlined) used for amplification of the promoter fragments
fw: GCTAGCAACTCGGTGGCCCTCTTAAC (*Nhe*I)
rev: CCATGGACAGACACGAGCTCGCAAAAT (*Nco*I)
Inverse orientation
fw: CCATGGAACTCGGTGGCCCTCTTAAC
rev: GCTAGCACAGACACGAGCTCGCAAAAT

Table 4.1: *NR3C1* specific primers
Primer designed for genotyping and for amplification of the promoter fragments for subsequent ligation into the pGl3 vector

4.3.2 Luciferase assay

Reporter gene plasmids were created by cloning four 458 bp PCR fragments amplified from the 5'-flanking region of *GR*. Amplicons covered a region from nucleotide position 142763782 – 142763325 on chromosome 5. Primer-specific *Nhe*I- and *Nco*I recognition sites were added to the PCR products (see Table 4.1). Sequencing confirmed that the amplified fragments were identical with the genomic *GR* sequence for both the T and C alleles. PCR-fragments were *Nhe*I- and *Nco*I digested and subsequently ligated into the appropriate sites of pGL3-Basic luciferase reporter plasmid, cut by the same enzymes. Both constructs were ligated in sense (pGl3-T; pGl3-C)- and antisense directions (pGl3-Tinv; pGl3-Cinv).

4.3.3 Transient transfection and luciferase assays

U373MG glioblastoma-astrocytoma cells (ECACC 89081403) were grown in minimal essential Eagle's medium (Biowest), supplemented with 10 % foetal calf serum (FCS), 2 mM L-glutamine, 1 % penicillin/streptomycin (Pen/Strep) and 1 % non essential amino acids (NEAA) at 37°C in a humified atmosphere at 5 % CO_2. SK-N-SH human neuroblastoma cells (ECACC 86012802) were grown under the same conditions using RPMI1640, supplemented with 10 % FCS and 1 % Pen/Strep. U373MG Cells were transfected by using Superfect transfection reagent (Qiagen) in a ratio of 5:1 (µl Superfect / µg DNA) and with a ratio of 10:1 for SK-N-SH. In brief, cells were passaged at ~ 80 % confluency and spread in a density of 8 x 10^4 cells / ml for U373MG cells and 1 x 10^5 cells / ml for SK-N-SH, respectively, in 24 well plates. After 24 h of incubation, 0.8 µg *Firefly* luciferase constructs together with 0.2 µg pGL4.74 ([HRLUC/TK], *Renilla* Luciferase; Promega) in the presence of 5 µl (10 µl for SK-N-SH) Superfect and 20 µl serum free medium were added to the cell culture. The plasmid expressing *Renilla* luciferase was used to control for transfection efficiency. Promoter activity was tested under basal condition and after the addition

of 50 µM forskolin, 2 µM phorbol 12-myristate acetate (PMA), and 50 µM dexamethasone, added 24 hours after transfection. As internal control, pGL3-control and empty pGL3-basic vectors were transfected separately. Cells were harvested in 50 µl Passive Lysis Buffer (PLB, Promega) 48 h after transfection. *Firefly* and *Renilla* luciferase activities were determined sequentially with 10 µl of total cell lysates in 50 µl of luciferase assay reagent followed by addition of 50 µl of Stop & Glo reagent (Promega) per reaction. Chemiluminescence was measured for 10 sec in a liquid scintillation spectrophotometer (Berthold).

Experiments were performed in quadruplicates and derived from four independent experiments. Promoter activities are presented as Relative Light Units (RLU`s), calculated as the quotient of the individual *Firefly* luciferase driven value (promoter activity) divided by the corresponding Renilla luciferase driven value (transfection efficiency control).

4.3.4 Statistical Analyses

First, one-way analyses of variance (ANOVAs) were computed separately for the two investigated cell lines to compare transcriptional activity under basal conditions with activity after stimulation (dexamethasone, forskolin or PMA). Posthoc analyses were performed with LSD tests. In a second set of one-way ANOVAs the differences in transcriptional activity between the two alleles (C vs T) in all conditions (basal, dexamethasone, forskolin and PMA) and in both cell lines were compared. Linkage disequilibrium among the five SNPs was estimated with D' and r^2 using HaploView and only haplotypes with a frequency over two percent were included in the analyses. Unless otherwise stated statistical analyses were performed using SPSS 16.0 (Chicago, IL, USA). All results shown are the mean ± standard error of mean (SEM).

4.3.5 Supplementary Reanalysis

The subjects genotyped for the rs10482605 SNP were extensively phenotyped for characteristics of HPA axis regulation. The new genotype information was now added and all previously performed association analyses were repeated. A well established laboratory stress protocol, the Trier Social Stress Test (TSST; Kirschbaum et al. 1993) was used to assess ACTH and cortisol responses to psychosocial stress, and a low-dose (0.25 mg) dexamethasone suppression test was conducted to characterize HPA axis feedback mechanisms. The associations between endocrine responses to the TSST and dexamethasone suppression were reanalyzed using the same methods as reported in (Kumsta et al. 2007).

4.4 Results

4.4.1 Reporter gene assay

Luciferase activity is indicated in Figure 4.1 for C and T allele promoter constructs under unstimulated and stimulated conditions. Reporter gene experiments were performed in two brain derived cell lines (U373MG and SK-N-SH).

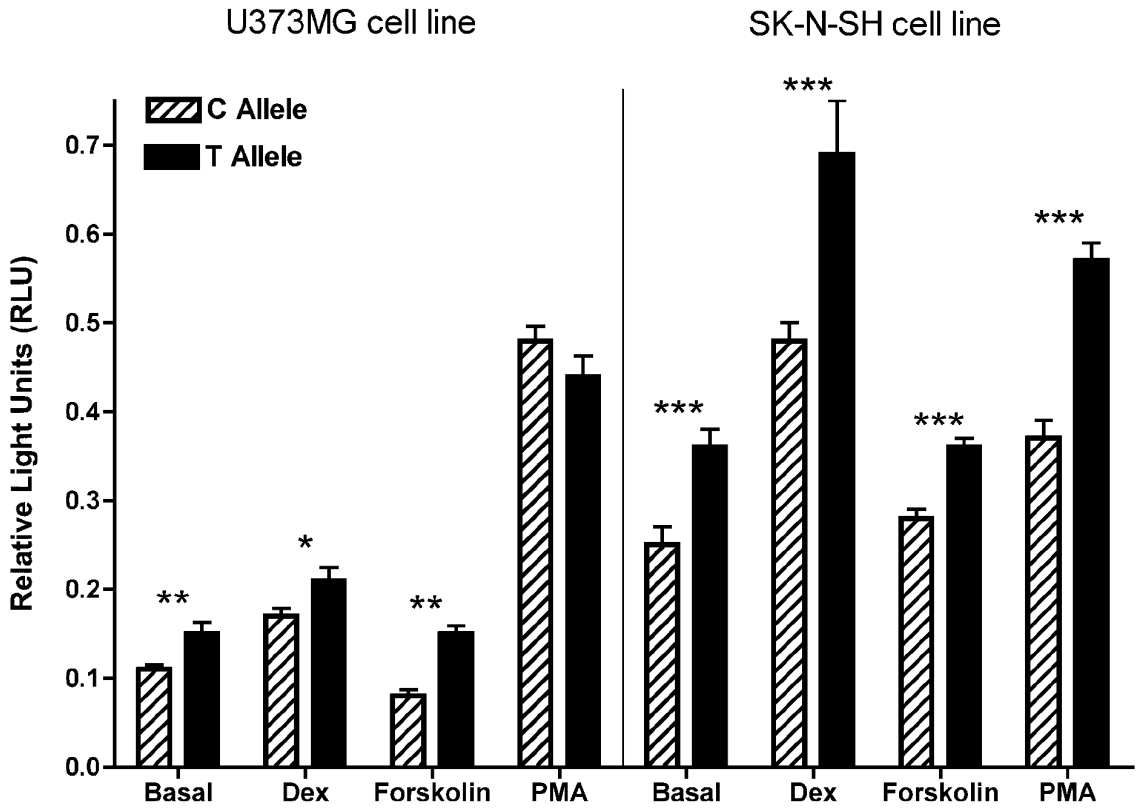

Figure 4.1: Gene expression activity of *NR3C1* C vs T allele promoter constructs
Gene expression activity of C vs T allele promoter constructs of rs10482605 under different signalling conditions in two brain derived cell lines (*p<.05; **p<.01: ***p<.0001)

In both cell lines, transcriptional activity increased for both the C and the T allele construct after stimulation with dexamethasone (Dex) and PMA (U373MG, C allele: $F_{3,47}$=3.13, p<.0001, post hoc: basal vs Dex and basal vs PMA both p<.0001; T allele: $F_{3,47}$=39.90, p<.0001, post hoc: basal vs Dex p=.08 and basal vs PMA p<.0001; SK-N-SH cell line: $F_{3,43}$>42.05 and all p<.0001 for

post hoc comparisons basal vs Dex and basal vs PMA for both alleles), while forskolin stimulation left activity unchanged (Post-hoc basal vs forskolin p>.05 in both cell lines for both alleles). Comparison of transcriptional activity between the alleles in the U373MG cell line revealed that under unstimulated conditions (basal) as well as under stimulation with dexamethasone and forskolin, the T allele showed significantly higher transcriptional activity (basal: $F_{1,23}$=8.47, p<.008; Dex: $F_{1,23}$=4.47, p<.046; Forskolin: $F_{1,23}$=15.37, p<.001). No significant differences could be observed under PMA stimulation in this cell line ($F_{1,23}$=2.03, p=0.2). In the SK-N-SH cell line, even more pronounced, activity of the T allele was significantly higher under all conditions (all $F_{1,23}$>22.32, all p<.0001).

4.4.2 Haplotype structure

Genotyping of 219 subjects using 5 markers revealed a novel haplotype structure of the *GR*. Figure 4.2, only showing haplotypes with a frequency over two percent, indicates that the minor

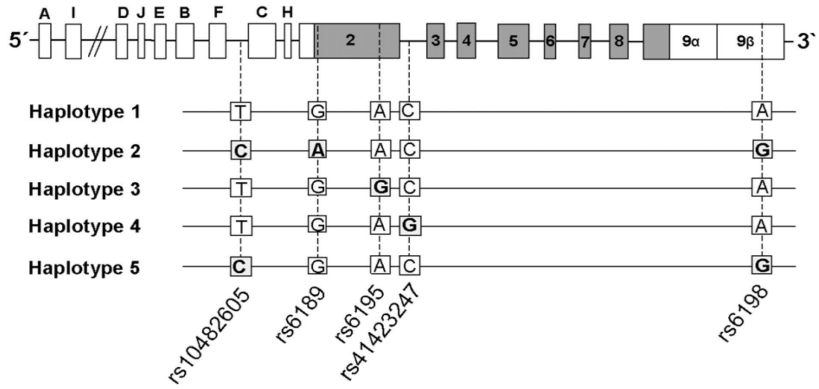

Figure 4.2: *NR3C1* haplotype structure
Genomic organization of the human glucocorticoid receptor gene (*GR*) is indicated in the upper part of the Figure. Boxes indicate exons and the coding region of the gene is drawn in dark shade. 5´region of the gene shows multiple exons 1 (capital letters A-I) according to Turner and Muller (2005) and Presul et al. (2007). The rs10482605 SNP is located 366 bp from the transcription start site of alternative exon 1C. The lower part of the Figure shows the observed haplotype structure and frequencies. Minor alleles are denoted by bold letters.

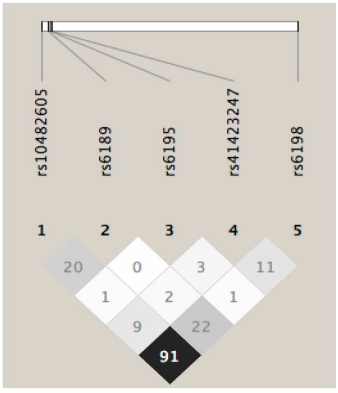

Figure 4.3: *GR* LD-structure
Linkage disequilibrium (LD) structure of *GR* showing r^2 values (r^2 colour scheme) and localisation of the 5 SNPs analysed.

C allele of SNP rs10482605 (NR3C1-1) is restricted to previously described Haplotypes 2 and 5. Linkage disequilibrium (LD) between the SNPs is shown in Table 4.2, indicating very high linkage between the rs10482605 C allele and the G allele of SNP rs6198 in exon 9beta. Figure 4.3 graphically represents the LD plot generated by HaploView.

4.4.3 Reanalysis of association with HPA axis activity phenotypes

One aim of our study was to reanalyze the association between *GR* haplotypes and endocrine responses to psychosocial stress as well as to dexamethasone administration, taking into account the newly genotyped promoter SNP in our phenotyped cohort. Since the LD analysis showed that

L1	L2	D'	LOD	r^2
NR3C1-1	R23K	0.926	9.06	0.2
NR3C1-1	N363S	1.0	1.47	0.012
NR3C1-1	*Bcl*I	0.948	9.5	0.098
NR3C1-1	**9beta A/G**	**0.965**	**56.03**	**0.915**
R23K	N363S	1.0	0.51	0.0030
R23K	*Bcl*I	1.0	1.93	0.025
R23K	9beta A/G	1.0	11.19	0.229
N363S	*Bcl*I	1.0	2.97	0.039
N363S	9beta A/G	1.0	1.48	0.012
*Bcl*I	9beta A/G	1.0	11.96	0.111

Table 4.2: *NR3C1* specific linkage disequilibrium
LD between the five investigated loci (L1=locus 1, L2=locus 2, LOD=logarithm of odds);
NR3C1-1: rs10482605, R23K: rs6190, N363S: rs6195, *Bcl*I: rs41423247, 9beta A/G: rs6198

the C allele falls entirely on two previously analyzed haplotypes no new (but an extended) haplotype has to be taken into account. Therefore, the statistical results of our previous (Kumsta et al. 2007) and the current association analyses are identical and will not be presented in detail. Briefly, the strongest associations were observed for male carriers of Haplotype 5 harbouring the newly analyzed SNP, showing significantly higher ACTH responses to the TSST ($F_{1,56}$=5.57, p=.02) and a relative non-suppression of ACTH following dexamethasone administration ($F_{1,56}$=17, p<.0001). Consistently, a trend was observed for enhanced cortisol responses to the TSST ($F_{1,56}$=2.39, p=.1). Although no new patterns of associations emerged after the reanalysis, the finding of increased endocrine responses to the TSST and the results from the dexamethasone suppression test in male carriers of Haplotype 5 can be reassessed from a functional perspective, given the newly described haplotype structure and results on gene expression analysis of the rs10482605 SNP.

4.5 Discussion

Genetic polymorphisms located in the 5´-regulatory gene region potentially influence the amount of translated gene product via differentially expressed levels of mRNA. We functionally characterized a common SNP (rs10482605) located in the promoter region of *GR* that was previously associated with major depressive disorder. Analyses of transcriptional activity comparing C and T allele promoter constructs revealed that, in two CNS tumor cell lines, the C allele showed lower transcriptional activity. Luciferase expression was analyzed under different signalling conditions and in the SK-N-SH cell line, the C allele showed significantly lower activity under unstimulated conditions as well as under all stimulation conditions, i.e. under dexamethasone, forskolin and PMA. In the U373MG cell line, similar results could be observed except that after PMA no differences in transcriptional activity between the alleles were revealed.

Recent analysis of the 5' flanking region of the human *GR* revealed several transcripts, which are based on at least nine alternative exon 1s (Presul et al., 2007; Turner and Muller, 2005). Each alternative exon is under control of its individual proximal promoter region which likely explains tissue-specific *GR* expression (Turner et al. 2006). Transcripts containing exon 1C and 1B mRNA show the broadest tissue distribution, and expression via promoters 1B and 1C seems to underlie constitutive expression of the *GR*. Here, we investigated a SNP located in the promoter region of alternative exon 1C. Extensive analyses of exon 1C promoter region revealed several transcription factor binding sites (five sites for Sp1, one for AP-2; Nobukuni et al. 1995) and one for Ying Yang 1 (YY1; Breslin and Vedeckis, 1998). Additional putative binding sites for Early Growth Response (EGR-1) are present. No binding of transcription factors has been experimentally confirmed at the locus of the investigated SNP. In silico analysis using Transfac 10.1® did not reveal differential binding of mammalian transcription factors between the two alleles. Possibly, binding of an unknown *cis*- or *trans*-acting factor is involved in allele specific

promoter activity. Future *in vitro* studies using DNA footprinting or electrophoretic mobility shift assays are warranted to identify allele specific transcription factor binding.

Genotyping of 219 individuals showed that the C allele of the promoter SNP (rs10482605) and the 9beta G allele (rs6198) are in high linkage disequilibrium. The 9beta A/G SNP was first characterized by DeRijk et al. (2001) and functional studies revealed a stabilizing effect of this polymorphism on GRbeta mRNA *in vitro*, possibly leading to enhanced expression of GRbeta protein. GRbeta is one of several GR protein isoforms and is generated through an alternative splicing pathway linking further downstream sequences of exon 9, termed exon 9beta, to the end of exon 8. In contrast to the functionally active and most abundant isoform GRalpha (Hagendorf et al. 2005), GRbeta is unable to bind ligand, is transcriptionally inactive (Yudt et al., 2003) and exerts a dominant negative effect on transactivation by GRalpha. Several mechanisms underlying this dominant negative effect have been described (Charmandari et al., 2005; Oakley et al., 1999; Oakley et al., 1996).

Results of our linkage analysis show that two functional SNPs fall on this newly characterized haplotype. The C allele of the rs10482605 SNP, located in the promoter region, leads to decreased levels of GR mRNA and the G allele of rs6198, located in the 3'-untranslated region, leads to relatively increased levels of GRbeta mRNA. We suggest that presence of this haplotype leads to a reduced quantity of functional GRalpha protein and to a relatively higher level of GRbeta protein, potentially increasing the GRbeta/GRalpha ratio. The expected effects on systemic level would be a relative GC resistance, due to the functional characteristics of the GRbeta protein isoform. This assumption is supported by a number of findings. For instance, enhanced expression of the GRbeta was associated with GC resistance in allergic disease (Hamid et al., 1999; Leung et al., 1997; Sousa et al., 2000), and a study with rheumatoid arthritis patients showed a higher prevalence of the 9beta G allele in patients than in controls (Derijk et al., 2001).

Furthermore, a lower transrepressing activity has been reported for the GRbeta protein variant (van den Akker et al., 2006b) and a lower risk of persistent *Staphylococcus aureus* nasal carriage in the 9beta GG genotype was observed, possibly resulting from reduced GC-induced immune suppression (van den Akker et al. 2006). Further support for a relative GC resistance in carriers of the 9beta G allele comes from the observation of reduced central obesity in women, and a more favourable lipid profile in male G allele carriers (Syed et al. 2006). Recently it was shown that the 9beta G carrying haplotype was associated with the risk of cardiovascular disease, possible due a more active proinflammatory system indicated by higher C-reactive protein and interleukin 6 levels (van den Akker et al. 2008). Lastly, our own data demonstrate sex-specific association between 9beta AG genotype and HPA axis regulation, with male 9beta AG carriers showing increased ACTH and cortisol secretion following exposure to a psychosocial stress protocol and a relative non-suppression of ACTH after a low dose (0.25 mg) dexamethasone suppression test (Figure 4.4). The sex by genotype interactions are discussed elsewhere in detail (Kumsta et al. 2007).

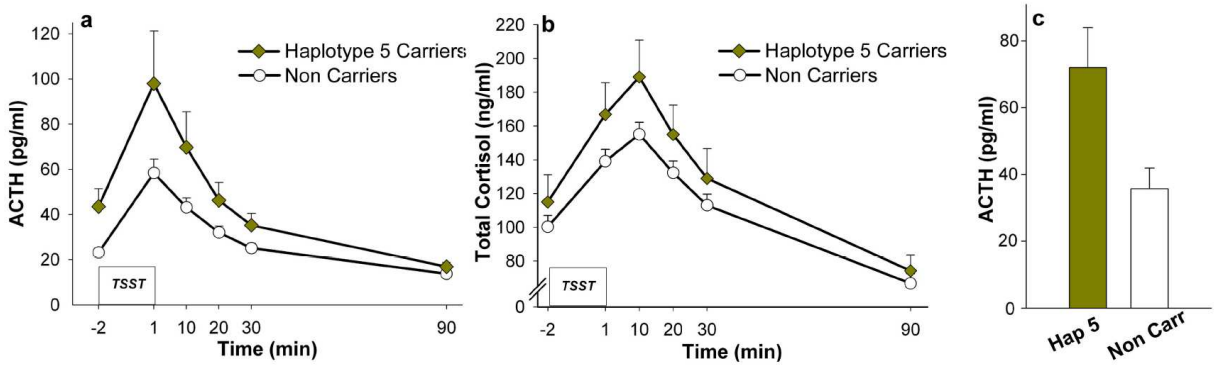

Figure 4.4: ACTH and cortisol profiles in male subjects

a) ACTH and b) cortisol responses to the Trier Social Stress Test and c) 8:00 am ACTH levels after 0.25 mg overnight Dexamethasone in male Haplotype 5 (rs10482605 TC / rs6198 AG) carriers carriers compared to male non-carriers. Since genotyping for the rs10482605 SNP did not result in new haplotypes, the effects shown here are identical to the effect reported before (Figure modified from Kumsta et al., 2007).

The role of altered GR function in major depression is very well established (Holsboer, 2000; Pariante, 2006). The association between the rs10482605 SNP and major depression can be reassessed in light of the functional results and the newly described haplotype structure provided in the present study. As laid out above, a relative GC resistance would be expected in carriers of Haplotype 5 and this would be consistent with the notion of altered GR function and/or reduced GR mRNA number presumed to underlie HPA axis hyperactivity in some patients with major depression. However, in the study by van West et al. (van West et al. 2006), the association between rs10482605 and major depression in a Belgian sample was driven by a higher number of C allele carriers in the control group and - on haplotype level - by a higher frequency in controls of a haplotype corresponding to Haplotype 5 in our study. The association observed in the Swedish sample was driven by a higher number of 22/23EK carriers on a genotype level and by a higher frequency of a haplotype (corresponding to Haplotype 2 in our study) in the depressed group. Despite the well established role of altered GC signalling in major depressive disorder, the question if changes in HPA axis regulation are a cause or a consequence of depression has not been resolved (Neigh and Nemeroff 2006). It could be speculated that in carriers of Haplotype 5, the increased HPA axis responses to stress are due to the relative inherited GC resistance and that the HPA axis is merely regulated at a higher set point.

Furthermore, it has to be pointed out that it is not possible to directly associate enhanced HPA axis responses following a psychosocial stress protocol – as observed in male carriers of Haplotype 5 in our healthy sample (Kumsta et al. 2007) – to vulnerability of developing a major depressive episode. The question if carriers of a GC resistance conferring haplotype are at increased or decreased risk of psychopathology and if this vulnerability is related to HPA axis responses to psychosocial stress should now be studied in patients in acute and remittent phases of depression.

Glucocorticoid signalling is known to be highly tissue specific (van West et al. 2006). Therefore, the functional consequences of the investigated SNP might vary in different cell types, for instance due to differential expression of transcription factors and other cofactors involved in gene regulation. Analysis of gene expression of our constructs was tested in brain derived tumor cells only and not in peripheral cells. However, as central GR signalling is involved in HPA axis regulation and since changes in central GR signalling are assumed in major depressive disorder, the investigation of brain derived cells seems a plausible choice.

We suggest that carriage of Haplotype 5 possibly leads to lower levels of total GR due to reduced expression conferred by the promoter SNP as shown in this study, and to a relative increase in GRbeta protein levels due to presence of the 9beta A/G SNP as shown in the study by DeRijk et al. (2001) The main goal of this study was to investigate functionality of the promoter SNP and to further characterize *GR* haplotype structure, and RNA samples (from peripheral blood cells) were unfortunately not available. Therefore, GRbeta mRNA levels could not be assessed.

In summary, we functionally investigated a common *GR* SNP that has been associated with major depression in two brain derived cell lines. It could be demonstrated that the minor C allele of rs10482605 had lower transcriptional activity under unstimulated and different stimulation conditions *in vitro*, possibly leading to a reduction in the amount of expressed GR *in vivo*. Furthermore, strong linkage disequilibrium between the C allele of rs10482605 and the G allele of the 9beta A/G SNP (rs6198) extends a previously described *GR* haplotype and allows a reinterpretation of previously reported associations. We suggest that Haplotype 5 harbours two functional SNPs with additive effects, leading to a relative GC resistance on systemic level, which may significantly contribute to the individual vulnerability for the development of stress related disorders, including major depression.

5. General Discussion

5.1 Synopsis

During the past decades, a large number of genes, chromosome aberrations, mutations and polymorphisms have been identified, possibly contributing to the development of psychiatric diseases (Caspi et al. 2002; de Kloet et al. 1998; Hariri et al. 2002; Holsboer 2000; Kumsta et al. 2007; Lesch et al. 1996; Meyer et al. 2005; Preisig et al. 2000; Rotondo et al. 2002). Nevertheless, the complex phenomena present in psychiatric diseases cannot readily be explained by classic genetic features such as DNA mutations and/or polymorphisms in susceptible genes alone. To date, psychiatric genetic research has focused mainly on linking specific chromosomal regions to particular mental illness or aspects of these illnesses, drug response, sub- or endophenotypes. However, it has proven difficult to find robust genetic associations. Mental illnesses are complex disorders caused by the interplay of multiple genes and environmental factors. Recent findings could demonstrate that environmental factors can modulate DNA transcription via epigenetic mechanisms (Abdolmaleky et al. 2004; Dolinoy et al. 2006; Mill et al. 2008; Petronis 2003; Petronis et al. 2000; Szyf et al. 2008; Tsankova et al. 2007; Weaver et al. 2004). Epigenetics can be regarded as the interface at which environmental stimuli and internal factors interact with gene expression. It may also help to explain MZ twin discordance, as genetically identical individuals were shown to display varying levels of not only disease-relevant gene expression (Fraga et al. 2005; Petronis et al. 2003). Furthermore, epigenetic mechanisms such as gene imprinting or X-chromosome inactivation have been shown to play a crucial role in regulating allelic expression levels in the mammalian genome (Chen et al. 2001; Heijmans et al. 2007; Hogart et al. 2007; Jiang et al. 2008; Murrell et al. 2004). Therefore, it can be hypothesized that many features of complex traits, which are observed in mental diseases, might be explained by the dynamic structure of epigenetic hallmarks as well as their reversibility, heritability and time and tissue specificity.

The studies presented here show how genetic polymorphisms and/or epigenetic modification may play a role in affecting human behavior and possibly, mental health. Furthermore, these studies provide evidence of how SNPs by themselves, and/or possibly in combination with epigenetic modification, might explain inter-individual variations in human behavior and mental pathologies by linking genetic polymorphisms with social and chemical environmental exposures to behavioral and physiological outcomes.

5.2 *SLC12A6* promoter investigation

The study presented in chapter 2, demonstrating functional analysis of different *SLC12A6* promoter alleles was based on studies identifying linkage for periodic catatonia on chromosome 15q14. After fine mapping and mutation screening of candidate loci, genes such as connexin 36 (*CX36*, Meyer et al. 2002a) and nicotinic alpha7 receptor (*CHRNA7*, Meyer et al. 2002b) failed to identify disease-causing mutations. In 2005 two rare G/A polymorphisms located in *SLC12A6* 5'regulatory region, which were associated with bipolar disorder in a case control study were identified (Meyer et al. 2005).

There has been a long-lasting debate between scientists following the Common Disease-Common Variant approach (CDCV) and those supporting the Common Disease-Rare Variant (CDRV; Doris 2002) approach studying complex inheritance of psychiatric disorders. The CDCV approach is based on the assumption that for most complex inherited disorders, such as BPD, MDD or SCZ, inheritance is multifactorial with several genetic variants interacting with each other. Furthermore, the underlying genotype is supposed to be influenced by the environment and can lead to disease causing circumstances under certain conditions. As the diseases are relatively common, showing similar phenotypes in the affected individuals, it has been postulated that the causative variants are common, too. However, no common genetic variant has been shown to be related to any psychiatric disease conclusively, even now. This might be explained by the high prevalence of these genetic variants and by the difficulties in

the identification of the variants functionalities as each one of them are neither sufficient nor obligatory to cause the disease.

On the other hand, supporters of the CDRV approach propose that disease-causing variants rather could be identified by the use of special outlier cases, such as extreme phenotypes (periodic catatonia), patients with chromosome aberrations, or the study of large multiplex families. The basic assumption is to find a variant of major affect, contributing to a large extend to the etiology of the respective phenotype which can be identified by simple Mendelian inheritance. Supporters of this approach are aware that these mutations or polymorphisms are probably rare and are not appropriate to explain most cases of diseases but harbouring the opportunity for the identification genes, involved in yet unknown pathophysiological pathways that are shared by more common forms of the disorder (Kohn 2005).

The findings on the functionality of the upstream *SLC12A6* 32418760 G/A promoter SNP demonstrated here support the CDRV approach for the identification of complex inherited disorders. Performing linkage analysis for periodic catatonia using 12 multiplex families, linkage to *SLC12A6* was confirmed in one family (family 11; see Meyer et al. 2005) by mutation screening which identified two polymorphic G/A sites in the *SLC12A6* 5' region. Bisulfite specific promoter investigation revealed methylation of the 5' adjacent cytosine at the allelic *SLC12A6* 32418760G promoter site but not the *SLC12A6* 32418760A site. Functionality of this promoter SNP introducing a methylation site was demonstrated by performing *luciferase* reporter gene assays. Under all conditions assayed, the *SLC12A6* G-allelic constructs were showing significantly decreased promoter activity compared to the *SLC12A6* A-allelic constructs. These findings confirm the results described by Meyer et al. (2005), suggesting *SLC12A6* as one of the susceptibility genes responsible for the pathogenesis of bipolar disorder and perhaps, schizophrenia with bipolar features. However, an intriguing finding is that mutations or rare polymorphisms in the same gene can lead to

different forms of neuropsychiatric disorders. Mutations in *SLC12A6* leading to a non-functional protein have been described as autosomal recessive causative for ACCPN, whereas findings on *SLC12A6* promoter polymorphisms rather point to an autosomal dominant inheritance (see family 11; Meyer et al. 2005). As already postulated by Kohn (2005), this might be explained by the variants location in the *SLC12A6* promoter region. Variant promoter genotypes and epigenotypes, as described in chapter 2, can cause changes in gene expression and may lead to neurodevelopmental disorder, which is progressive with age. However, the findings of different allelic *SLC12A6* promoter activities cannot conclusively exclude the possibility of another disease causing mutation distally to the *SLC12A6* locus. Furthermore, this is supported by findings in family 9 (Meyer et al. 2002b), pointing to linkage between markers D15S1042 and D15S182, which are located distally of *SLC12A6*. Taken together, findings on altered *SLC12A6* promoter activity presented here support the findings of Meyer et al (2005), but replication of the association study in a larger sample is warranted. At the same time, it would be highly interesting to identify homozygote carriers of the *SLC12A6* G-specific allele which have not been found yet, possibly due to the low frequency of these rare variants. As already postulated in chapter 2, consequences of the polymorphisms on *SLC12A6* mRNA expression levels or alternative first exon usage should also be investigated in *post mortem* brain tissue comparing *SLC12A6* G and A allele carriers.

In principle, we could demonstrate that the *SLC12A6* upstream G /A SNP can lead to allele specific methylation and, that the presence of the G allele is associated with reduced promoter activity– possibly brought about by methylation. *In silico* analysis did not reveal altered transcription factor binding sites comparing A and G-allele, but it cannot be ruled out that any unknown *cis*- or *trans*-acting factors may affect allele specific promoter activity. As the upstream palindromic sequence TTAC**A**TT is abolished in the presence of the rare G nucleotide (TTAC**G**TT), putative transcription factor binding efficiency could be altered and

also be fine-tuned by cytosine methylation in a time, tissue and environment dependent manner (Figure 5.1a).

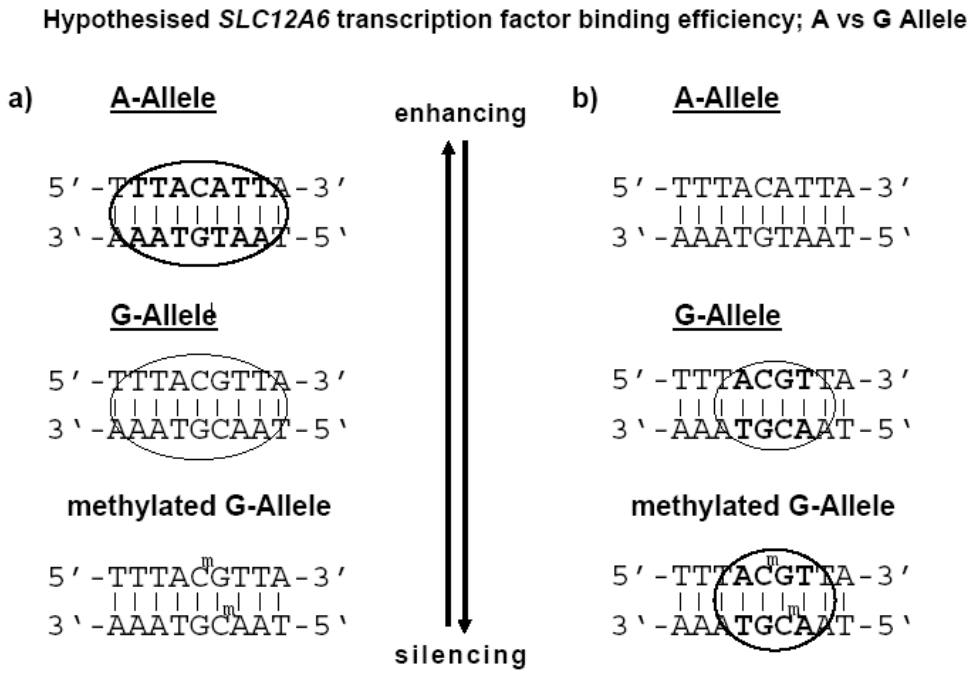

Figure 5.1: a) suggested enhanced binding efficiency of a positive regulator of *SLC12A6* gene transcription at the A polymorphic site; b) suggested higher binding efficiency of a negative regulator in the *SLC12A6* G-allele in dependence of DNA methylation; thickness of the circles indicating hypothesized altered transcription factor binding efficiency. Palindromic sequences are indicated in bold letters.

On the other hand, it was hypothesized that methylated DNA can facilitate histone deacetylation by recruiting chromatin remodelling enzymes such as histone deacetylases (HDACs) via the action of methyl-CpG binding domain proteins (MBDs) like MeCP2 (Tsankova et al. 2007). In the *SLC12A6* G-allele, a palindromic sequence of sense and antisense strand is created (5’-AC**G**T-3’), possibly representing a MBD binding site. Furthermore, this putative protein binding site could be modulated by cytosine methylation leading to enhanced MBD binding and decreased promoter activity (Figure 5.1b). For both possibilities, higher binding efficiency for a positive regulator of gene transcription in the A-

allele or higher binding efficiency of a negative regulator in the G-allele might help explaining the putative causative effect of this variant in family 11 (Meyer et al. 2005).

As already postulated in chapter 2, SNPs introducing or abolishing methylation sites in genes regulatory regions could lead to altered binding efficiency of silencing or enhancing transcription factors. Statistically, SNPs can be expected at a frequency of about 300 bp across the genome (Goldstein and Cavalleri 2005; Kingsmore et al. 2008), thus findings as described for *SLC12A6* possibly can be expected to occur in regulatory regions of nearly every gene. Recently, identification of a new SNP (rs25531 G/A), located upstream of the 5-HTTLPR, was reported (Nakamura et al. 2000), to lead to lowered promoter activity in the G-allele, which causes an altered transcription factor binding efficiency, possibly modified by DNA methylation of the cytosine adjacent to the variant guanine. Meanwhile, altered binding efficiency for the activator protein 2 transcription factor (AP-2), which is believed to be a critical factor in regulating neural gene expression in mammals was reported (Kraft et al. 2005). Interestingly, *in silico* analysis revealed that in the presence of the rs25531 C nucleotide a methylation site is created which could efficiently modify AP-2 binding. Unfortunately, methylation at this site could not be demonstrated by analyzing the lymphocyte genomic DNA of *SLC6A4* G-allele carriers at the present moment (Moser et al., unpublished observations). However, using tissues harbouring differential methylation pattern, such as frontal cortex and germlines, as already demonstrated (Mill et al. 2008), it cannot be ruled out that the *SLC6A4* allelic G-site will also be found to be methylated with putative consequences for brain development and stress response. However, hypothesised methylation at the *SLC6A4* rs25531 G-site could represent a susceptibility locus for the linkage of DNA variation with epigenetic modification for life.

A similar finding was also found in the *TPH2* promoter, where the allelic cytosine of rs7963717 A/C was found to be methylated (Moser et al., unpublished observations). However, functionality of the *TPH2* rs7963717 C nucleotide has still to be demonstrated, and

further investigation of this SNP is warranted, as a binding motif for the human upstream transcription factor 1 (*USF1*) is created in the C-allelic site. Furthermore, it was demonstrated by site-directed mutagenesis and methylation protection studies that the core palindromic sequence (CA**C**GTG; the variant cytosine of rs7963717 is highlighted) is essential for USF-binding and is strongly reduced by the cytosine methylation of the central CpG dinucleotide (d'Adda di Fagagna et al. 1995). These observations support the possibility that SNPs can be functional not only by themselves, but also enable or restrain the possibilities for epimutations. As these epimutations are susceptible to environmental factors, they could functionally link life experiences with epigenetic modification and thus, with behavioral traits.

In silico analysis of the mouse (*M. musculus*; UCSC mm9 assembly, July 2007) and the rats (*R. norvegicus*; UCSC rn4 assembly, Nov. 2004) *Slc12a6* promoter regions revealed polymorphic regions, such as rs13463364 T/G in the murine *Slc12a6* promoter, which also leads to SNP dependent allelic methylation sites. In a future study, one could breed animals of different *Slc12a6* allelic promoter genotypes and compare homozygote animals of genotypes prone to alternative methylation with animals less prone to *Slc12a6* promoter methylation. Animals could also be tested in differential settings after experiencing differential maternal behavior, differential nutrition or pharmacological treatment such as TSA or 5-aza treatment. Behavioral traits could then be compared to epigenetic *Slc12a6* promoter methylation pattern in various brain tissues, and also to the levels of *Slc12a6* mRNA transcripts, with additional respect to possible alternative first exon usage. Furthermore, for animals which show abnormal behavioral phenotypes, they could also be tested after the administration of antidepressants and antipsychotic drugs. However, these experiments could only lead to a better understanding of the putative consequences of altered *Slc12a6* promoter activities but not to explain the consequences of the variants found in human. This can be explained by the

different SNP localisation in the *Slc12a6/SLC12A6* promoters and thus, the different transcription factor binding and structural chromatin organization between the animals and humans.

5.3 *NR3C1* promoter investigation

In chapter 3, findings on *NR3C1* 1F specific promoter methylation pattern in human hippocampus are presented. This study was based on findings by Weaver et al. (2004) demonstrating *Nr3c1* promoter methylation pattern being dependent on maternal care. Other studies also demonstrated the epigenotype to be dependent on environmental conditions such as different life-styles and nutrition. We hypothesized to find different inter-individual *NR3C1* 1-F specific methylation pattern by investigating *post mortem* hippocampus tissue derived from 32 unrelated individuals. Our finding of an almost unmethylated *NR3C1* 1-F promoter points towards differences in glucocorticoid receptor promoter methylation between human and rats. As already described in chapter 3, our investigation is limited by the availability of human *post mortem* tissue, exclusively deriving from older individuals and also by the lack of data on early childhood, critical life events, medication etc. However, we believe that it is unlikely that all subjects experienced the same environmental conditions, resulting in a highly similar epigenotypes as observed. Therefore, we hypothesized that *NR3C1* 1F promoter in aged humans is constitutively unmethylated. Excluding an age-dependent effect, it would be highly interesting to investigate Nr3c1 1_7 epigenotype for hippocampi deriving from aged rats too. Furthermore, investigation of human *post mortem* tissue from younger donors is warranted. If possible, these tissues should derive from patients with ascertained psychopathological disorders such as major depression compared with control subjects (e.g. victims of accidents vs. subjects committing suicide). In this context, studies looking for similar methylation pattern of peripheral tissues compared to distinct brain areas will be more than welcome. If similar epigenetic organization could be identified in peripheral tissue

compared to distinct brain regions, research would no longer be restricted to the investigation of *post mortem* brain tissue. One could then analyse, for example, DNA methylation pattern of premature birth children, grown up in an incubator during their first weeks of life compared to those of normal birth- or even better, if possible, comparing twin pairs, triplets or quadruplets, where not all siblings had incubator experience.

Furthermore, it could be a challenging animal experiment to investigate rats with differential experienced maternal care in a "learned-helplessness model". According to the literature, one could postulate differences in depressive-like symptoms dependent on experienced maternal care. In analogy to a study by Caspi and colleagues (2003) in which an individual's response to environmental insults is moderated by his or her genetic makeup, it could be highly challenging to investigate whether a certain *Nr3c1*-specific epigenotype could function as a protective or susceptibility factor for the development of helplessness in rats.

Apart from epigenetic modification, genetic variation in the genome also takes many forms. In the study presented in chapter 4, we could demonstrate the impact of a SNP located in the 5' regulatory region of the glucocorticoid receptor gene on promoter activity using an *in vitro* experimental design. In contrast to the CDRV approach described in chapter 2, this study was based on the CDCV theory. Furthermore, the use of *NR3C1* as a candidate gene for association studies is not based on linkage analysis but on its physiological role in the regulation of stress. Common variants with small effects, potentially altering the binding efficiency for *cis*- or *trans*-acting factors such as rs10482605 T/C, and located in the *NR3C1* promoter region of alternative exon 1C, could also contribute to the individual's health. Hyperactivity of the HPA axis as observed in many patients with major depression is normalized by antidepressants medication (Holsboer 2000; Pariante 2004). Several studies in cellular and animal experimental systems have shown that antidepressants can enhance GR function and increase GR expression (Pariante et al. 2003; Pepin et al. 1989; Seckl and Fink

1992; Yau et al. 2004). GR expression in the study presented in chapter 4 was tested under three different stimulation conditions inducing different intracellular signalling cascades. Forskolin, which increases intracellular levels of cyclic AMP, did not increase promoter activity (consistent with the lack of a cAMP-responsive element site in the promoter region of exon 1C). Luciferase expression was increased for both variant alleles under dexamethasone and PMA stimulation. However, promoter activity was still significantly higher in the T allele under dexamethasone (in the U373MG cell line) and under both dexamethasone and PMA (in the SK-N-SH cell line). Mood stabilizers such as lithium and valproic acid have been shown to directly affect signal transduction cascades (Manji and Lenox 1998) and the stimulation of GR expression has been shown for various tricyclic antidepressants and SSRIs (Juruena et al. 2004). Therefore, it could be speculated that carriers of the investigated SNP react differentially to antidepressant treatment as has been shown for another the ER22/23EK polymorphism (van Rossum et al. 2006). This could be tested in future *in vitro* studies including preincubation with different classes of antidepressants.

5.4 Epigenetics and psychiatric disorders

The role of epigenetic factors in the origin of diseases has been demonstrated in cancer. For example, aberrant DNA methylation patterns are being explored for, as biomarkers for cancer detection, assessment of prognosis and prediction of response to therapy. DNA methylation inhibitors as well as HDACs have been shown to be effective in silencing tumors. By analogy, pharmacological therapies in mental illness are likely to benefit from an epigenetic perspective. For example, drug therapy in mental disorder treatment is known to be effective only after some weeks of drug administration. For example, this has been observed for lithium and valproic acid, hypothesizing that the effects may be mediated by altered gene transcription. On the other hand, the altered gene transcription may be caused by altered gene promoter organization. Furthermore, this theory is supported by recent findings showing that

the effects of non-selective monoamine oxidase inhibitors, such as tranylcypromine or the antipsychotic drug haloperidol, may be mediated via histone demethylation/deacetylation (Karagiannis et al. 2006; Lee et al. 2006). Additionally, it can be argued that environmental and (epi-) genetic factors can also affect the maturation of brain circuits involved in affective function, and ultimately, increasing the likelihood of mental disorders in adulthood.

The epigenetic model of psychiatric diseases according to Arturas Petronis (Petronis 2004) is based upon three general principles. First, like the DNA sequence, the epigenome of somatic cells is mitotically inherited, but unlike the DNA sequence, the methylation profile is highly dynamic. The epigenetic status of the genome is cell type-specific, developmentally regulated and influenced by intrinsic genomic and also environmental factors. Second, as epigenetic processes are involved in the regulation of gene expression, epimutations can have a high impact on phenotypic effects. Epimutations can lead to harmful silencing or over-expression of certain genes being causative for a certain phenotype. Thus, epigenetic status can affect the individual's outcome without any change in the underlying DNA primary sequence, such as mutations or disease predisposing polymorphisms. Third, some epigenetic signals, rather than being reset and erased during gametogenesis, could be transmitted meiotically across generations (Richards 2006). This has obvious ramifications for the identification of the molecular substrate of inherited predispositions, in which heritable phenotypic variation is assumed to result exclusively from DNA-sequence variants. In this context, it is highly interesting that Mill et al. (2008) identified changes in DNA-methylation pattern in cells of the prefrontal cortex as well as in germ cells being associated with schizophrenia and bipolar disorder. Furthermore, they found evidence that psychosis-associated DNA methylation was similarly altered in different brain regions, involved in glutamatergic and GABAergic neurotransmission and brain development. By performing gene-ontology analysis, they identified regional distinctions of DNA methylation in genes involved in mitochondrial function, brain development, and stress response. As they also detected decreased epigenetic

modularity in both the brain and the germline of affected individuals, they suggested that systemic epigenetic dysfunction may be associated with major psychosis. These findings are supported by the data of the *SLC12A6* promoter presented here, where the additional methylation site only present in the G-allele may influence the individual's outcome by changing *SLC12A6* specific expression in a cell, developmental and environmental specific way beginning directly after fertilization.

As Szyf et al. (2008) suggested, epigenetic programming of gene expression is stable and long-term but yet responsible and responsive. A change in epigenotype could have the same impact as a genetic polymorphism leading to either enhancing or silencing of gene expression. Differences in epigenotypes could thus lead to different phenotypes. Recent data provide evidence that the DNA methylation pattern in the hippocampus is responsive to environmental exposures even in the adult. It was shown by another group (Miller et al. 2007; Miller and Sweatt 2007), that DNA methylation/ demethylation events are involved in learning processes in the hippocampus. It was speculated that epigenetic mechanisms serve as a medium for the adaptability of the genome and thus of the organism to altered environments during life. The well established relationship between socioeconomic status and physical health might be mediated by factors influencing DNA disposability and there is growing evidence that social environment might sculpt our genome through modifying our epigenome. The impact of DNA methylation on gene activity has been explained by two different mechanisms in literature - the "critical site of methylation" model and the "methylation density" model. The significance of the first model is underlined by the data on *SLC12A6* SNP dependent methylation pattern presented here. The critical site of methylation model emphasises that methylation of specific cytosines modulates transcription factor binding and thus promoter activity and gene expression. The "methylation density" model of methylation is supported by the *NR3C1* 1F promoter methylation in human post mortem hippocampus tissue described here. Whereas the basic study performed in rats (Weaver et al. 2004) is

representing a combination of both models - critical site and density of methylation - human *post mortem* tissue was harbouring an almost unmethylated 1F specific *NR3C1* promoter (Moser et al. 2007).

6. Outlook

Differences in the human genome depend not only on single nucleotide exchanges but also range from large, microscopically visible chromosome anomalies as described for *DISC1* (Millar et al. 2001; Millar et al. 2000), abundance of submicroscopic copy number variation (Redon et al. 2006), to gene x environment interaction (Caspi et al. 2002; Caspi et al. 2003). However, the challenge will be to use and to combine the most advanced methods and technologies from, if possible, all highly specified, scientific areas and to integrate all the data in order to draw a complete picture of how the healthy and ill brain work. Furthermore, it reflects a growing evidence that complex illnesses will no longer be solely tractable to traditional genetic linkage approaches that assume Mendelian dominant recessive inheritance of a single major gene influencing a trait (Risch 1990a; Risch 1990b; Risch 1990c; Risch 1990d; Risch and Merikangas 1996). Therefore, the Diagnostic and Statistical Manual of Mental Disease (DSM-IV for the fourth and current edition) and the International Statistical Classification of Diseases and Related Health Problems (ICD, 10th revision, 2007), both very helpful in the clinical practice, will have to consider the needs of laboratory investigation and define more discrete (endo-) phenotypes. It is assumed that genetic determination of endophenotypes is likely to be relatively less complex than that of the illness overall phenotype. Thus, the genetic investigation of distinctly defined endophenotypes may be more promising for the genetical analysis of behavioral determinants in future.

6.1 The Diagnostic and Statistical Manual of Mental Disease (DSM)

The Diagnostic and Statistical Manual of Mental Disease, a very helpful tool in the clinical practice, is considered to have little biological validity as the diagnostic criteria give name to clusters of symptoms that overlap with each other. For example, schizophrenia, bipolar disorder, anxiety, depression, and attention deficit and hyperactivity as defined by DSM-IV, share many symptoms, and clear clinical phenotypization has proven difficult. For instance,

periodic catatonia according to Leonhard (1999) is conceptualized as a subtype of schizophrenia. However, in the current Diagnostic and Statistical Manual of Mental Disorders, periodic catatonia is not recognized as a separate disorder but is associated with psychiatric conditions such as schizophrenia (catatonic type), bipolar disorder, post-traumatic stress disorder, depression and other mental disorders, as well as drug abuse and/or overdose. According to DSM, as long as individuals are categorized to the same disorder but hardly share any common feature or symptom, and as long as subphenotypes are conceptualized to belong to one disorder but also occur in other disorders, it will stay cumbersome or even futile to identify the disease causing genomic traits. Therefore, another categorization for DSM-V or DSM-VI which introduce endophenotypes as diagnostic criteria is warranted. Using endophenotypes such as biomarkers of specific symptoms, drug response and brain imaging, rather than a DSM label, could become a very helpful tool and emerging model for the scientific investigation of mental diseases. Ideally, endophenotypes have monogenic roots; but it is likely that many also have polygenic bases. Therefore, endophenotypes should be chosen carefully and fulfilling the following criteria (Petronis et al. 2003): Endophenotypes have to be associated with the illness in the whole population. They should be heritable and manifestable, whether the illness is active or not. Endophenotypes and illness should co-segregate within families and the endophentype in non-affected family members should be found at a higher rate than in the general population.

Phenotypes and endophenotypes could be analyzed and investigated by using the instruments described in the following section in order to draw a complete picture of the various neuronal dysfunctions underlying and influencing mental diseases.

6.2 Brain Imaging/ Imaging Genomics

“Imaging genetics”, a novel research strategy, attempts to identify gene effects on a brain systems level and has provided significant contributions to the understanding of the complex

impact of hereditary factors on psychiatric illness. As mental illness and normal brain function has a hereditary component, it is essential that the genes relating to different brain development, mental function and dysfunction have to be considered. The integration of the imaging data with the rich genetic data resulting from the human genome project will enhance the understanding of brain functions and mental illnesses.

Hariri et al. (Hariri et al. 2002) were the first to combine advanced technologies in order to investigate regional brain activation in dependence of different underlying genotypes. They applied a functional magnetic resonance imaging (fMRI) paradigm comparing homozygous carriers of the *SLC6A4* long and short-allele (Lesch et al. 1996). After presentation of fearful stimuli they found higher responses in amygdala activation in carriers of the *SLC6A4* short promoter allele, findings which were replicated in the meantime by other groups (Canli et al. 2006; Furmark et al. 2004; Pezawas et al. 2005).

Performing imaging genomics for *SLC12A6* and *NR3C1* also would be of highest interest. To test individuals of different *SLC12A6* promoter alleles in a brain imaging setting could represent a highly sophisticated experiment. As loss of function mutations in the *SLC12A6* lead to Andermann Syndrome with or without agenesis of the *corpus callosum* (ACCPN), it would be challenging to investigate whether allelic *SLC12A6* lowered promoter activity leads to altered callosal connectivity or even altered *corpus callosum* size.

Another potential study design could measure neuronal activity, especially in the hippocampus, in dependence of *NR3C1* genotype and also putative *NR3C1* epigenotype. Neuronal activity could be measured in hippocampal structures of individuals with or without experienced childhood adversity, thus extending a study which describes the modulating effect of maternal care on hippocampal size (Buss et al. 2007).

6.3 Global variation in copy number

Recently, genetic variations in the human genome ranging from kilobases (kb) to megabases (Mb) in size have been described (Iafrate et al. 2004; Sebat et al. 2004). Deletions, insertions, duplications and complex multi-site variants of more than 1 kb in length are collectively termed copy number variations (CNVs). It is thought that CNVs cover approximately 12 % of the human genome, potentially altering gene dosage, disrupting genes or perturbing regulation of their expression, even at long range distances (Nguyen et al. 2006; Repping et al. 2006). Thus, variations in copy number can cause disease or confer risk to complex disease traits by modulating phenotypic variation and adaptability (Inoue and Lupski 2002; Shaw-Smith et al. 2004). Appearance of CNVs is highly ubiquitious and complex. Different copy number profiles were shown in monozygotic twins concordant as well as discordant for certain phenotypic features (Bruder et al. 2008), highlighting the importance of CNVs for the study of genetic causes to diseases.

Loss of heterozygosity (LOH) can occur with or without CNVs. Clinical importance for CNVs introducing LOH could be demonstrated in cystic fibrosis, in which a CNV in the cystic fibrosis transmembrane conductance regulator-gene (*CFTR*) leads to the loss of one allele, thereby unmasking a functional mutation in the other allele shown to be responsible for the disease. In tumors, regions of LOH frequently indicate the presence of an altered tumor suppressor gene. LOH can also influence disease progression by altering a region affected by imprinting, as commonly seen in Prader-Willi Syndrome, Angelman Syndrome and Wilms' Tumor. There is growing evidence that CNVs are possibly contributing to a multitude of phenotypes and diseases; however, traditional methods such as linkage analysis often failed to detect CNVs as they also cannot detect epimutations. How CNVs may be used in future is illustrated by two recent reports showing that a fraction of autism risk loci is likely to involve rare CNVs rather than SNPs (Sebat et al. 2007; Szatmari et al. 2007). Analogous to *DISC1*, identified by performing linkage analysis, these studies show how the investigation of CNVs

could lead to the identification of genes that carry disease causing polymorphisms. Therefore, the contribution of CNVs, especially in combination with putative alteration of the resulting epigenotypes, remains to be elucidated. During the next years, an effort in discovering most of the common and rare copy number variants in the human population can be expected. This will enable systematic exploration of SNPs and CNVs in combination with altered epigenotypes to identify contributors in the aetiology of common disorders and also complex, multifactorial traits.

6.4 Genome wide association studies (GWAS)

With the completion of the Human Genome Project in 2003 and the foundation of the Hap Map project in 2005, researchers now have additional tools to investigate genetic disorders. For comparison: Human genetic mapping was initially based on the restriction fragment length polymorphism (RFLP) analysis. In order to construct a linkage map of the human genome sequence, variations in the beginning were laboriously assayed by Southern blots (Botstein et al. 1980; Donis-Keller et al. 1987; Lander and Botstein 1986a; Lander and Botstein 1986b). This approach was further refined using the linkage desiquilibrium (LD) mapping. This procedure implies that a mutation is shared by affected individuals through common descent and will be surrounded by shared alleles at nearby loci, thus representing the haplotype of the ancestral chromosome on which the mutation first occurred. Human LD mapping flourished as RFLP mapping was replaced by the PCR-based microsatellite analysis in the 90s of the last century (Weissenbach et al. 1992).

Genome scans were carried out for a multitude of common diseases and traits of complex inheritance but failed to identify robust, reproducible genetic loci. As it has been proven difficult to identify high-frequency polymorphisms with small effects by performing family linkage studies it was argued that association studies should be more powerful (Risch and Merikangas 1996). During the last 10 years, more than 10 million single nucleotide

polymorphisms have been identified (Goldstein and Cavalleri 2005; Kingsmore et al. 2008). Furthermore, the International HapMap project has genotyped approximately 4 million common SNPs (occurring with a minor-allele frequency of more than 5%) in human populations and has assembled these genotypes computationally into a genome-wide map of SNP-tagged haplotypes (Consortium 2005). These resources, together with array technologies for massively parallel SNP genotyping and the well-established epidemiological case-control association studies, have rendered GWAS feasible. Whereas the first generation of GeneChip® arrays provided possibilities to analyse more than 10.000 SNPs (Kennedy et al. 2003; Matsuzaki et al. 2004a; Matsuzaki et al. 2004b), the new Affymetrix® Genome-Wide Human SNP Array 6.0 features for 1.8 million genetic markers, including more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation at the same time.

Genome-wide association studies examine SNPs across the genome, and they represent a promising way to study complex and common diseases in which many genetic variations contribute to a person's risk. This approach has already identified SNPs related to several complex conditions including diabetes, Parkinson disease, heart disorders, obesity, Crohn's disease and prostate cancer, as well as genetic variations that influence response to anti-depressant medications (Consortium 2007; Ioannidis et al. 2007; Zeggini et al. 2008). Using GWAS, it can be assumed that a lot of genetic loci will be identified, contributing to psychiatric (endo-) phenotypes. Furthermore, as proteins interact with one another within genetic networks modulating cellular programming, specific phenotypes are generated and the identification of specific SNP combinations underlying a certain phenotype can be expected. Additionally, GWAS will also help to understand the interactions between a person's genetic makeup and the environment (gene x environment interactions), the interplay of proteins (gene x gene interactions) and also the biochemical effects of specific drugs.

Other chip-based advanced technologies will further support the investigation of DNA regulation. Gene expression arrays, ChIP-on-chip analysis and oligonucleotide tiling arrays will allow the investigation of a person's tissue-specific, whole genome DNA-methylation pattern, histone-acetylation status, transcription factor binding density and the resulting gene expression very quickly and efficiently.

As the region on chromosome 15q11-15 already has shown association with autism, bipolar disorder, Prader-Willi syndrome (Jiang et al. 2008) due to differential methylation (Hogart et al. 2007; Schanen 2006), and/or copy number polymorphisms (CNPs), SNPs and also the association of the allelic methylation of *SLC12A6* presented here, are forcing further studies on chromosome 15 by the use of all the techniques aforementioned.

6.5 The Large-Scale DNA Sequencing

DNA sequencing is and will become an increasingly important part of genetic analysis, with significant consequences not only for basic research but also for fundamental biological processes such as gene expression, gene regulation, and phylogenetics, and for applied research such as diagnostics, forensics, and gene therapy. Sequencing technology have come a long way from the first DNA sequencing reports (Gilbert and Maxam 1973) of a 24 base pair sequence of the lac-operator. The introduction of the Sanger sequencing method (Sanger et al. 1977) revolutionized and dramatically sped up the process and accuracy of DNA sequencing. At the beginning of this century, about 50 years after Watson and Crick first described the biochemical structure of the DNA (Watson and Crick 1953), the sequence that makes up the human genome was proclaimed (Consortium 2004; Venter 2003). In the meantime, the entire genomic sequence of two single persons, namely James Watson and Craig Venter, was reported. James Watson's sequence revealed 7.093 non-synonymous SNPs (nsSNPs), with 2889nsSNPs thought to be benign, 522 possibly disease-producing, 286 probably damaging and 201unknown variants. In order to reduce the costs, the large scale

genome sequencing is already used for the analysis of pooled DNA samples. Using the 454 Life Science sequencing technology, over 3.000 novel nsSNPs were identified in a pool of 20 individuals (Albert et al. 2007). It is thought and expected that this new technology represents the beginning of a true genomic medicine, based on a complete genetic sequence of an individual. Compared to DNA Chip technology (Illumina®; Affymetrix®), large scale DNA sequencing is also suitable for the discovery of novel mutations, rare SNPs or other polymorphisms, such as insertions or deletions. However, the challenge will still be to associate these variants with a specific phenotype or predisposition.

The identification and collection of the individual's DNA impact of information may be welcomed but caution is warranted to the hypothesis-free collection of sequence information. For example, a study performed on some 44.000 twin pairs concluded that environmental, and not inherited genetic factors, were the main determinants of sporadic cancer (Lichtenstein et al. 2000). The identification of the human genome in 2004 represented a dramatic breakthrough in genetic research. However, the challenge since then was, still is and will be to gain insights into that 4 letter primary structure. Still, far from all genomic properties being identified, even lesser is known about their functions or regulation patterns. Additionally, the complexity in the understanding of the human genome was enhanced as after intense investigation of only 1% of the human genome. The ENCODE researchers in 2007 found evidence that most of the human genome is transcribed as RNA at some time or another. Furthermore, they found lots of strikingly conserved regions located far from any genes, indicating that they have some important function (Birney et al. 2007). Therefore, not only the investigation of the genome, but of the transcriptome and proteome, as well as the regulation of the enormous changes occurring in an organism between conception and death, represent the big challenge for genetic research in future. As Thomas C. Erren (Erren et al. 2008) formulated: "Our lives are unrepeatable experiments and every second a myriad external

factors interact with genetic and epigenetic factors and with chance to determine whether we are well or ill, smart or dull, successes or failures".

6.6 Animal studies

Starting from a defined phenotype in the widely used "top-down" approaches in psychiatric genetics, performing linkage and association studies, the physiological roles of the genes identified often remain poorly understood. During the past decades, a number of *in vitro* techniques have been established to get a better understanding of the protein-protein interactions. In order to better understand the physiologic functionality of proteins, it is now more common to use a procedure known as "Gene Knockout". The mouse has been chosen as a model organism for gene knockouts for several reasons: as a mammal, the mouse shares many similarities with the human not only in development, body plan and physiology but also in behavior and diseases. Additionally, 99% of the mouse genes have human orthologues and the mouse genome supports targeted mutagenesis in specific genes by homologous recombination in embryonic stem cells, allowing genes to be altered efficiently. This additional genetic tool, which was developed in the late 1980s, has led to many insights into human biology and diseases (Chen et al. 2001; Murphy and Lesch 2008).

For instance, a genomic region located on chromosome 22 harbouring *MLC1* was found to be highly associated with periodic catatonia in studies of multiplex families (Meyer et al. 2001; Stober et al. 2000). The investigation of the *MLC1* knockout mouse (Meyer et al. in prep) will be of highest interest, (i) to investigate the animals in behavioral tests, (ii) to identify further association of *MLC1* with catatonic features, and (iii) to elucidate the physiological role of MLC1. Thus, the combination of these comparative human and mouse studies may extend the opportunities to examine genetic alterations from a "bottom-up" approach, which is complementary to the traditional "top-down" genetic approach based upon studies of individuals with diagnosed neuropsychiatric disorders and their family members.

6.7 Conclusion

The recent findings on the considerable plasticity of the human genome and epigenome, a field still in its infancy, might help to explain phenotypic discrepancies in the penetrance of genetic traits and/or in the severity of the resulting phenotypes. Most epidemiological, clinical and molecular features associated with mental disorders, such as discordances between monozygotic twins and temporal remission of affections are difficult to explain with traditional gene and environment based approaches. Other complexities of psychiatric disorders include fluctuating course of disease, with periods of remission and relapse, cycling between manic and depressive states, sexual dimorphism, peaks of susceptibility to disease coinciding with major hormonal rearrangements, and parent of origin effects. These observations have led to the epigenetic theory of mental disorders reflecting the importance of epigenetic factors in mediating susceptibility to distinct mental conditions (Abdolmaleky et al. 2004; Dolinoy et al. 2006; Jirtle and Skinner 2007; Peedicayil 2003; Petronis et al. 2000; Szyf et al. 2008).

In conclusion and to emphasise the complexity and importance of DNA methylation in particular, and epigenetics in general , I would like to refer to Dana Dolinoy (Dolinoy et al. 2007) proposing:

"… if the genome is compared to the hardware in a computer, the epigenome is the software that directs the computer's operation".

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Appendix

Eidesstattliche Erklärung Trier, im Mai 2008

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und keine anderen als die angegebenen Hilfsmittel benutzt wurden.

_____

Dirk Moser